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(57) Abstract

There are provided Cutinase variants of a parent Cutinase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved. In particular, the compatibility to anionic surfactants has been improved by reducing the binding of anionic surfactants to the enzyme.

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MODIFIED CUTINASES, DNA, VECTOR AND HOST

TECHNICAL FIELD

The present invention generally relates to the field of lipolytic enzymes. More in particular, the invention is concerned with lipolytic enzymes which have been modified by means of recombinant DNA techniques, with methods for their production and with their use, particularly in enzymatic detergent compositions.

BACKGROUND AND PRIOR ART

Lipolytic enzymes are enzymes which are capable of hydrolysing triglycerides into free fatty acids and diglycerides, monoglycerides and eventually glycerol. They can also split more complex esters such as cutin layers in plants or sebum of the skin. Lipolytic enzymes are used in industry for various enzymatic processes such as the inter- and trans-esterification of triglycerides and the synthesis of esters. They are also used in detergent compositions with the aim to improve the fat-removing properties of the detergent product.

The most widely used lipolytic enzymes are lipases (EC 3.1.1.3). For example, EP-A-258 068 and EP-A-305 216 (both Novo Nordisk) both describe production of fungal lipases via heterologous host micro-organisms by means of rDNA techniques, especially the lipase from Thermomyces lanuginosus/Humicola lanuginosa. EP-A-331 376 (Amano) describes lipases and their production by rDNA techniques, and their use, including an amino acid sequence of lipase from Pseudomonas cepacia. Further examples of lipases produced by rDNA technique are given in WO 89/09263 and EP-A-218 272 (both Gist-Brocades). In spite of the large number of publications on lipases and their modifications, only the lipase from Humicola lanuginosa has so far found wide-spread commercial application as additive for detergent products under the trade name Lipolase (TM).

A characteristic feature of lipases is that they exhibit interfacial activation. This means that the enzyme activity is much higher on a substrate which has formed

interfaces or micelles, than on fully dissolved substrate. Interface activation is reflected in a sudden increase in lipolytic activity when the substrate concentration is raised above the critical micel concentration (CMC) of the substrate, and interfaces are formed. Experimentally this phenomenon can be observed as a discontinuity in the graph of enzyme activity versus substrate concentration.

The mechanism of interfacial activation in lipases has been interpreted in terms of a conformational change in the protein structure of the lipase molecule. In the free, unbound state, a helical lid covers the catalytic binding site. Upon binding to the lipid substrate, the lid is displaced and the catalytic site is exposed. The helical lid is also believed to interact with the lipid interface, thus allowing the enzyme to remain bound to the interface.

WO-A-92/05249 (Novo Nordisk) discloses genetically modified lipases, in particular the lipase from Humicola lanuginosa, which have been modified at the lipid contact zone. The lipid contact zone is defined in the application as the surface which in the active form is covered by the helical lid. The modifications involve deletion or substitution of one or more amino acid residues in the lipid contact zone, so as to increase the electrostatic charge and/or decrease the hydrophobicity of the lipid contact zone, or so as to change the surface conformation of the lipid contact zone. This is achieved by deleting one or more negatively charged amino acid residues in the lipid contact zone, or substituting these residues by neutral or more positively charged amino acids, and/or by substituting one or more neutral amino acid residues in the lipid contact zone by positively charged amino acids, and/or deleting one or more hydrophilic amino acid residues in the lipid contact zone, or substituting these residues by hydrophobic amino acids.

Cutinases are a sub-class of enzymes (EC 3.1.1.50), the wax ester hydrolases. These enzymes are capable of degrading cutin, a network of esterified long-chain fatty acids and fatty alcohols which occurs in plants as a protective coating on leaves and stems. In addition, they

possess some lipolytic activity, i.e. they are capable of hydrolysing triglycerides. Thus they can be regarded as a special kind of lipases. Contrary to lipases, however, cutinases do not exhibit any substantial interfacial activation.

Cutinases can be obtained from a number of sources, such as plants (e.g. pollen), bacteria and fungi. Because of their fat degrading properties, cutinases have been proposed as ingredients for enzymatic detergent compositions. For example, WO-A-88/09367 (Genencor) suggests combinations of a surfactant and a substantially pure bacterial cutinase enzyme to formulate effective cleaning compositions. Disclosed are detergent compositions comprising a cutinase obtained from the Gram negative bacterium Pseudomonas putida ATCC 53552. However, in the more recent European patent application EP-A-476 915 (Clorox), it is disclosed that the same enzyme - which is then referred to as a lipase - is no more effective than other lipases in removing oil stains from fabrics, when used by conventional methods.

Recently, the three-dimensional structure has been determined of a cutinase from Fusarium solani pisi (Martinez et al. (1992) Nature 356, 615-618). It was found that this cutinase does not possess a helical lid to cover the catalytic binding site. Instead, the active site serine residue appears to be accessible to the solvent. These findings appear to confirm the present theory about the mechanism of interfacial activation in lipases.

The cutinase gene from Fusarium solani pisi has been cloned and sequenced (Ettinger et al., (1987) Biochemistry 26, 7883-7892). WO-A-90/09446 (Plant Genetics Systems) describes the cloning and production of this gene in E. coli. The cutinase can efficiently catalyse the hydrolysis and the synthesis of esters in aqueous and non-aqueous media, both in the absence and the presence of and interface between the cutinase and the substrate. On the basis of its general stability, it is suggested that this cutinase could be used to produce cleaning agents such as laundry d tergents and other specialized fat dissolving preparations such as

cosmetic compositions and shampoos. A way to produce the enzyme in an economic feasible way is not disclosed, neither are specific enzymatic detergent compositions containing the cutinase.

Because of this characteristic feature, i.e. the absence of interfacial activation, we define for the purpose of this patent application Cutinases as lipolytic enzymes which exhibit substantially no interfacial activation. Cutinases therefore differ from classical lipases in that they do not possess a helical lid covering the catalytic binding site.

As mentioned above, only the lipase derived from Humicola lanuginosa has so far found wide-spread commercial application as additive for detergent products under the trade name Lipolase (TM). In his article in Chemistry and Industry 1990, pages 183-186, Henrik Malmos notes that it is known that generally the activity of lipases during the washing process is low, and Lipolase (TM) is no exception. During the drying process, when the water content of the fabric is reduced, the enzyme regains its activity and the fatty stains are hydrolysed. During the following wash cycle the hydrolysed material is removed. This also explains why the effect of lipases is low after the first washing cycle, but significant in the following cycles. Thus, there is still a need for lipolytic enzymes which exhibit any significant activity during the washing process.

We have found that Cutinases, in particular the cutinase from Fusarium solani pisi, exhibit a clear in-the-wash effect. However, there is still a need for Cutinase variants having improved in-the-wash lipolytic activity in anionic-rich detergent compositions, and for methods for producing such enzymes.

In accordance with the present invention, there are provided Cutinase variants wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved. More in particular, it was found that the lipolytic activity of eukaryotic Cutinases, more in particular of Cutinases from Fusarium

solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea, in anionic-rich detergent compositions may be improved by reducing the binding of anionic surfactants to the enzyme.

DEFINITION OF THE INVENTION

A Cutinase variant of a parent Cutinase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved, in particular by reducing the binding of anionic surfactants to the enzyme.

DESCRIPTION OF THE INVENTION

The invention relates to variants of Cutinase enzymes. As discussed above, Cutinases can be obtained from a number of sources, such as plants (e.g. pollen), bacteria and fungi. The Cutinase to be used as parent Cutinase or starting material in the present invention for the modification by means of recombinant DNA techniques, is chosen from the group of eukaryotic Cutinases. Eukaryotic Cutinases can be obtained from various sources, such as plants (e.g. pollen), or fungi.

The group of (eukaryotic) fungal Cutinases appears to comprise two families with different specificities, leaf-specificity and stem-specificity. Cutinases with leaf-specificity tend to have an acidic or neutral pH-optimum, whereas Cutinases with stem-specificity tend to have an alkaline pH-optimum. Cutinases having an alkaline pH-optimum are more suitable for use in alkaline built detergent compositions such as heavy duty fabric washing powders and liquids. Cutinase having an acidic to neutral pH-optimum are more suitable for light duty products or rinse conditioners, but also for industrial cleaning products.

In the following Table I, four different Cutinases with stem-specificity are listed, together with their pH-optima.

TABLE I

Examples of cutinases with stem-specificity	pH-optimum
Fusarium solani pisi	9
Fusarium roseum culmorum	10
Rhizoctonia solani	8.5
Alternaria brassicicola (PNBase I)	9

Especially preferred in the present invention are Cutinases which can be derived from wild type Fusarium solani pisi (Ettinger et al. 1987). When used in certain detergent compositions, this Cutinase exhibits clear "in-the-wash" effects.

Also suitable as parent Cutinase or starting material in the present invention for the modification by means of recombinant DNA techniques, are Cutinases having a high degree of homology of their amino acid sequence to the Cutinase from Fusarium solani pisi. Examples are the Cutinases from Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea. In Figure 11 the partial amino acid sequences of these Cutinases are shown and it can be seen that there is a high degree of homology.

Alternative to the improvement of Fusarium solani pisi cutinase by modification of its gene, genetic information encoding Cutinases from other eukaryotic organisms can be isolated using 5'- and 3'- DNA probes derived from Fusarium solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea cDNA encoding (pro)cutinase and probes recognizing conserved sequences in other Cutinases and if necessary, using these probes to multiply cDNA's derived from messenger RNA's (mRNA's) of Cutinase producing eukaryotic cells using the Polymerase Chain Reaction or PCR technology (see, for example WO-A-92/05249). After cloning and expression the thus obtained Cutinases encoding genes in E. coli according standard procedures, the Cutinases are tested on their performance in (fatty) soil removal under appropriate conditions. In this way a number of natural occurring variants of the above mentioned Cutinases can be obtained

with improved in-the-wash performance. Moreover, the sequences of these natural occurring Cutinases provide an excellent basis for further protein engineering of Fusarium solani pisi cutinase.

On the basis of new ideas about the factors determining the "in-the-wash" activity of lipolytic enzymes and careful inspection of the 3D structure of Fusarium solani pisi cutinase (Martinez et al. (1992) Nature 356, 615-618) and inspection of the 3D structure of Fusarium solani pisi cutinase we have found a number of possibilities how to improve the compatibility of this cutinase and Cutinases in general to anionic surfactants by means of recombinant DNA techniques.

Starting from the known 3D structure of the Fusarium solani pisi cutinase, the 3D-structure of the cutinase from Colletotrichum gloeosporiodes was obtained by applying rule-based comparative modelling techniques as implemented in the COMPOSER module of the SYBYL molecular modelling software package (TRIPOS associates, Inc. St. Louis, Missouri). The obtained model of the Colletotrichum gloeosporiodes cutinase was refined by applying energy minimization (EM) and molecular dynamics (MD) techniques as implemented in the BIOSYM molecular modelling software package (BIOSYM, San Diego, California). During EM and MD refinement of the model a knowledge-based approach was applied. The model was simultaneously optimized for the detailed energy terms of the potential energy function and known structural criteria. Model quality was assessed by criteria such as number and quality of hydrogen bonds, hydrogen bonding patterns in the secondary structure elements, the orientation of peptide units, the values of and main chain dihedral angles, the angle of interaction of aromatic groups and the sizes of cavities. Moreover, the model was checked for inappropriately buried charges, extremely exposed hydrophobic residues and energetically unfavourable positions of disulphide bridges. Relevant side-chain rotamers were selected from the Ponder & Richards rotamer library (Ponder et al. (1987) J.Mol.Biol. 193, 775-

791). The final choice of a particular side-chain rotamer from this library was based on structural criteria evaluations as mentioned above. MD was used to anneal the side-chain atoms into position. A similar approach was used to obtain the 3D-structure of the cutinase from Magnaporthe grisea.

The present invention shows that Cutinases can be modified in such a way that the interaction with anionic surfactants can be reduced without changing the "in-the-wash" performance of the modified Cutinase.

This may be achieved in a number of ways. First, the binding of anionic surfactants to the enzyme may reduced by reducing the electrostatic interaction between the anionic surfactant and the enzyme. For instance, by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolair tail of an anionic surfactant, by lysine residues. It is also possible to reduce the electrostatic interaction between the anionic surfactant and the enzyme shielding the positive charge of such an arginine residue by introducing within a distance of about 6 Å from said arginine a negative charge, e.g. an glutamic acid residue. Alternatively, the electrostatic interaction between the anionic surfactant and the enzyme may be reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by uncharged amino acid residues. Furthermore, the electrostatic interaction between the anionic surfactant and the enzyme may reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by negatively charged amino acid residues.

Another approach to reduce the binding between an anionic surfactant and the enzyme is to replace one or more amino acid residues which are located in a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by less hydrophobic amino acid residues. These

less hydrophobic amino acid residues are preferably selected from the group consisting of glycine, serine, alanine, aspartic acid and threonine.

Due to their improved anionics compatibility, the Cutinases variants produced according to the invention can bring advantage in enzyme activity, when used as part of an anionic-rich detergent or cleaning compositions. In the context of this invention, anionic-rich means that the detergent or cleaning composition contains a surfactant system which consists for more than 5%, generally more than 10%, and in particular more than 20% of anionic surfactants.

The Cutinase variants of the present invention were found to possess an improved in-the-wash performance during the main cycle of a wash process. By in-the-wash performance during the main cycle of a wash process, it is meant that a detergent composition containing the enzyme is capable of removing a significant amount of oily soil from a soiled fabric in a single wash process in a European type of automatic washing machine, using normal washing conditions as far as concentration, water hardness, temperature, are concerned. It should be born in mind that under the same conditions, the conventional commercially available lipolytic enzyme Lipolase (TM) ex Novo Nordisk does not appear to have any significant in-the-wash effect at all on oily soil.

The in-the-wash effect of an enzyme on oily soil can be assessed using the following assay. New polyester test having a cotton content of less than 10% are prewashed using an enzyme-free detergent product such as the one given below, and are subsequently thoroughly rinsed. Such unsoiled cloths are then soiled with olive oil or another suitable, hydrolysable oily stain. Each tests cloth (weighing approximately 1 g) is incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The wash liquor contains the detergent product given below at a dosage of 1 g per litre. The bottles are agitated for 30 minutes in a Miele TMT washing machine filled with water and using a normal 30°C main wash programme. The Cutinase variant is preadded to the wash liquor at 3 LU/ml. The control does not contain any enzyme.

The washing powder has the following composition (in % by weight):

LAS	6.9
Soap	2.0
Nonionic surfactant	10.0
Zeolite	27.0
Sodium carbonate	10.2
Sodium sulphate	13.0

After washing, the cloths are thoroughly rinsed with cold water and dried in a tumble dryer with cold air, and the amount of residual fat is assessed. This can be done in several ways. The common method is to extract the testcloth with petroleum ether in a Soxhlet extraction apparatus, distilling off the solvent and determining the percentage residual fatty material as a fraction of the initial amount of fat on the cloth by weighing.

According to a second, more sensitive method, brominated olive oil is used to soil the test cloths (Richards, S., Morris, M.A. and Arklay, T.H. (1968), Textile Research Journal 38, 105-107). Each test cloth is then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. A series of bottles is then agitated in a washing machine filled with water and using a normal 30°C main wash programme. After the main wash, the test cloths are carefully rinsed in cold water during 5 seconds. Immediately after the rinse, the test cloths dried in a dryer with cold air. After drying the amount of residual fat can be determined by measuring the bromine content of the cloth by means of X-ray. fluorescence spectrometry. The fat removal can be determined as a percentage of the amount which was initially present on the test cloth, as follows:

$$\% \text{ Soil removal} = \frac{\text{Bromine}_{bw} - \text{Bromine}_{aw}}{\text{Bromine}_{bw}} * 100\ \%$$

wherein: Bromine_{bw} denotes the percentage bromine on the cloth before the wash and Bromine_{aw} the percentage bromine after the wash.

A further method of assessing the enzymatic activity is by measuring the reflectance at 460 nm according to standard techniques.

In the context of this invention, a modified, mutated or mutant enzyme or a variant of an enzyme means an enzyme that has been produced by a mutant organism which is expressing a mutant gene. A mutant gene (other than one containing only silent mutations) means a gene encoding an enzyme having an amino acid sequence which has been derived directly or indirectly, and which in one or more locations is different, from the sequence of a corresponding parent enzyme. The parent enzyme means the gene product of the corresponding unaltered gene. A silent mutation in a gene means a change or difference produced in the polynucleotide sequence of the gene which (owing to the redundancy in the codon-amino acid relationships) leads to no change in the amino acid sequence of the enzyme encoded by that gene.

A mutant or mutated micro-organism means a micro-organism that is, or is descended from, a parent micro-organism subjected to mutation in respect of its gene for the enzyme. Such mutation of the organism may be carried out either (a) by mutation of a corresponding gene (parent gene) already present in the parent micro-organism, or (b) by the transfer (introduction) of a corresponding gene obtained directly or indirectly from another source, and then introduced (including the mutation of the gene) into the micro-organism which is to become the mutant micro-organism. A host micro-organism is a micro-organism of which a mutant gene, or a transferred gene of other origin, forms part. In general it may be of the same or different strain or species origin or descent as the parent micro-organism.

In particular, the invention provides mutant forms of the Fusarium solani pisi cutinase disclosed in WO-A-90/09446 (Plant Genetics Systems), and of the Cutinases from Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea. These Cutinase variants can be produced by a rDNA modified micro-organism containing a gene obtained or made by means of rDNA techniques.

Once the amino acid residues have been identified that should be replaced by another amino acid residue, for example mutation R17E relative to the sequence of Fusarium solani pisi cutinase or a homologue thereof.

It will be clear to the skilled man that such modifications will affect the structure of the Cutinase. Obviously, modifications are preferred which do not affect the electrostaic charge around the active site too much.The inventors have developed the necessary level of understanding of the balance between the inevitable distortion of the conformation of the enzyme and the benefit in increased enzyme activity, which makes is possible to predict and produce succesful Cutinase variants with a high rate of succes. In the following Table II and elsewhere in this specification, amino-acids and amino acid residues in peptide sequences are indicated by one-letter and three-letter abbreviations as follows:

TABLE II

A	=	Ala	= Alanine	V	=	Val	= Valine
L	=	Leu	= Leucine	I	=	Ile	= Isoleucine
P	=	Pro	= Proline	F	=	Phe	= Phenylalanine
W	=	Trp	= Tryptophan	M	=	Met	= Methionine
G	=	Gly	= Glycine	S	=	Ser	= Serine
T	=	Thr	= Threonine	C	=	Cys	= Cysteine
Y	=	Tyr	= Tyrosine	N	=	Asn	= Asparagine
Q	=	Gln	= Glutamine	D	=	Asp	= Aspartic Acid
E	=	Glu	= Glutamic Acid	K	=	Lys	= Lysine
R	=	Arg	= Arginine	H	=	His	= Histidine

In this specification, a mutation present in the amino acid sequence of a protein, and hence the mutant protein itself, may be described by the position and nature of the mutation in the following abbreviated way: by the identity of an original amino acid residue affected by the mutation; the site (by sequence number) of the mutation; and by the identity of the amino acid residue substituted there in place of the original. If there is an insertion of an extra amino acid into the sequence, its position is indicated by one or more subscript letters attached to the number of

the last preceding member of the regular sequence or reference sequence.

For example, a mutant characterised by substitution of Arginine by Glutamine in position 17 is designated as: Arg17Glu or R17E. A (hypothetical) insertion of an additional amino acid residue such as proline after the Arginine would be indicated as Arg17ArgPro or R17RP, alternatively as *17aP, with the inserted residue designated as position number 17a. A (hypothetical) deletion of Arginine in the same position would be indicated by Arg17* or R17*. The asterisk stands either for a deletion or for a missing amino acid residue in the position designated, whether it is reckoned as missing by actual deletion or merely by comparison or homology with another or a reference sequence having a residue in that position.

Multiple mutations are separated by plus signs, e.g. R17E+S54I+A128F designates a mutant protein carrying three mutations by substitution, as indicated for each of the three mentioned positions in the amino acid sequence. The mutations given in the following Table may be combined if desired.

The Table III given below shows certain useful examples of Cutinase variants according to the invention, based on the sequence of the Cutinases from Fusarium solani pisi, and Magnaporthe grisea.

TABLE III

Variants of Fusarium solani pisi cutinase:
R17L, R17K, R17E, L51A, L51S, R78L, T80D, R88E, R96N, R96Q, R156L, A195S, R196A, R196K, R196E.

Variants of Magnaporthe grisea cutinase:
A80D, A88E, R156L.

According to a further aspect of the invention, there is provided a process for producing the Cutinase variants of the invention. Naturally occurring Cutinase producing micro-organisms are usually plant pathogens and these micro-organisms are not very suitable to act as host cell for modified Cutinases genes. Consequently, the genes coding for modified (pro)Cutinases were integrated in rDNA

vectors that can be transferred into the preferred host micro-organism for rDNA technology. For this purpose rDNA vectors essentially similar to the rDNA vector described in WO-A-90/09446 can be used.

Naturally occurring Cutinase producing micro-organisms are not very suitable for fermentation processes. To improve the yield of the fermentation process a gene coding for improved Cutinases should be transferred into micro-organisms that can growth fast on cheap medium and are capable to synthesize and secrete large amounts of Cutinase. Such suitable rDNA modified (host micro-organisms) according to the present invention are bacteria, among others, Bacilli, Corynebacteria, Staphylococci and Streptomyces, or lower eukaryotes such as Saccharomyces cerevisiae and related species, Kluyveromyces marxianus and related species, Hansenula polymorpha and related species, and species of the genus Aspergillus. Preferred host micro-organisms are the lower eukaryotes, because these microorganisms are producing and secreting enzymes very well in fermentation processes and are able to glycolysate the Cutinase molecule. Glycosylation can contribute to the stability of the Cutinase in detergent systems.

The invention also provides genetic material derived from the introduction of modified eukaryotic Cutinase genes, e.g. the gene from Fusarium solani pisi, into cloning rDNA vectors, and the use of these to transform new host cells and to express the genes of the Cutinase variants in the new host cells.

Also provided by the invention are polynucleotides made or modified by rDNA technique, which encode such Cutinase variants, rDNA vectors containing such polynucleotides, and rDNA modified microorganisms containing such polynucleotides and/or such rDNA vectors. The invention also provides corresponding polynucleotides encoding the modified eukaryotic Cutinases, e.g. a polynucleotide having a base sequence that encodes a mature Cutinase variant, in which polynucleotide the final translated codon is followed by a stop codon and optionally having nucleotide sequences

coding for the prepro- or pro-sequence of this Cutinase variant directly upstream of the nucleotide sequences coding for the mature Cutinase variant.

In such a polynucleotide, the Cutinase-encoding nucleotide sequence derived from the organism of origin can be modified in such a way that at least one codon, and preferably as many codons as possible, are made the subject of 'silent' mutations to form codons encoding equivalent aminoacid residues and being codons preferred by a new host, thereby to provide in use within the cells of such host a messenger-RNA for the introduced gene of improved stability.

Upstream of the nucleotide sequences coding for the pro-or mature Cutinases, there can be located a nucleotide sequence that codes for a signal or secretion sequence suitable for the chosen host. Thus an embodiment of the invention relates to a rDNA vector into which a nucleotide sequence coding for a Cutinase variant or a precursor thereof has been inserted.

The nucleotide sequence can be derived for example from:

(a) a naturally occurring nucleotide sequence (e.g. encoding the original amino acid sequence of the prepro- or pro-cutinase produced by Fusarium solani pisi);

(b) chemically synthesized nucleotide sequences consisting of codons that are preferred by the new host and a nucleotide sequence resulting in stable messenger RNA in the new host, still encoding the original amino acid sequence;

(c) genetically engineered nucleotide sequences derived from one of the nucleotide sequences mentioned in preceding paragraphs a or b coding for a Fusarium solani pisi Cutinase with a different amino acid sequence but having superior stability and/or activity in detergent systems.

Summarizing, rDNA vectors able to direct the expression of the nucleotide sequence encoding a Cutinase gene as described above in one of the preferred hosts preferably comprise the following components:

(a) Double-stranded (ds) DNA coding for mature Cutinase or precutinase or a corresponding precutinase in which at least

part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell). In cases where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front. The translated part of the gene should always end with an appropriate stop codon;

(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the Cutinase (component (a));

(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the Cutinase (component (a);

(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,

(d2) an origin of replication suitable for the selected host;

(e1) Optionally a (auxotrophic) selection marker. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter;

(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the Cutinase in the host selected.

Such a rDNA vector can also carry, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the cutinase. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter region.

Another embodiment of this invention is the fermentative production of one of the various Cutinase variants described above. Such a fermentation can either be a normal batch fermentation, fed-batch fermentation or continuous fermentation. The selection of a process to be used depends on the host strain and the preferred down stream processing method (known per se). Thus, the invention also provides a process for producing a Cutinase variant as specified herein, which comprises the steps of fermentatively cultivating an rDNA modified micro-organism containing a gene made by rDNA technique which carries at least one mutation

affecting the amino acid sequence of the Cutinase thereby to confer upon the Cutinase improved activity by comparison with the corresponding parent enzyme, making a preparation of the Cutinase variant by separating the Cutinase produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the Cutinase varaint either from said broth or from said cells by physical or chemical concentration or purification methods. Preferably conditions are chosen such that the Cutinase variant is secreted by the micro-organism into the fermentation broth, the enzyme being recovered from the broth after removal of the cells either by filtration or centrifugation. Optionally, the Cutinase variant can then be concentrated and purified to a desired extent. These fermentation processes in themselves apart from the special nature of the micro-organisms can be based on known fermentation techniques and commonly used fermentation and down stream processing equipment.

Also provided by the invention is a method for the production of a modified micro-organism capable of producing a Cutinase variant by means of rDNA techniques, characterized in that the gene coding for the Cutinase variant that is introduced into the micro-organism is fused at its 5'-end to a gene fragment encoding a (modified) pre-sequence functional as a signal- or secretion-sequence for the host organism.

According to a further aspect of the invention, there are provided rDNA modified micro-organisms containing a Cutinase varaint gene and able to produce the Cutinase variant encoded by said gene. In an rDNA modified micro-organism, a gene (if originally present) encoding the native Cutinase is preferably removed, e.g. replaced by another structural gene.

According to a further aspect of the present invention, there are provided enzymatic detergent compositions comprising the Cutinase variants of the invention. Such compositions are combinations of the Cutinases variants and other ingredients which are commonly

used in detergent systems, including additives for detergent compositions and fully-formulated detergent and cleaning compositions, e.g. of the kinds known per se and described for example in EP-A-258 068.

The other components of such detergent compositions can be of any of many known kinds, for example as described in GB-A-1 372 034 (Unilever), US-A-3 950 277, US-A-4 011 169, EP-A-179 533 (Procter & Gamble), EP-A-205 208 and EP-A-206 390 (Unilever), JP-A-63-078000 (1988), and Research Disclosure 29056 of June 1988, together with each of the several specifications mentioned therein, all of which are hereby incorporated herein by reference.

The Cutinase variants of the present invention can usefully be added to the detergent composition in any suitable form, i.e. the form of a granular composition, a solution or a slurry of the enzyme, or with carrier material (e.g. as in EP-A-258 068 and the Savinase(TM) and Lipolase(TM) products of Novo Nordisk).

The added amount of Cutinase variant can be chosen within wide limits, for example from 10 - 20,000 LU per gram, and preferably 50 -2,000 LU per gram of the detergent composition. In this specification LU or lipase units are defined as they are in EP-A-258 068 (Novo Nordisk).

Similar considerations apply mutatis mutandis in the case of other enzymes, such as proteases, amylases, cellulases which may also be present. Advantage may be gained in such detergent compositions, where protease is present together with the Cutinase variant, by selecting such protease from those having pI lower than 10. EP-A-271 154 (Unilever) describes a number of such proteases. Proteases for use together with Cutinase variants can include subtilisin of for example BPN' type or of many of the types of subtilisin disclosed in the literature, e.g. mutant proteases as described in for example EP-A-130 756 or EP-A-251 446 (both Genentech); US-A-4 760 025 (Genencor); EP-A-214 435 (Henkel); WO-A-87/04661 (Amgen); WO-A-87/05050 (Genex); Thomas et al. J.Mol.Biol. (1987) 193, 803-813; Russel et al. Nature (1987) 328, 496-500.

The invention will now be further illustrated in the following Examples. All techniques used for the manipulation and analysis of nucleic acid materials were performed essentially as described in Sambrook et al. (1989), except where indicated otherwise.

In the accompanying drawings is:

Fig. 1A. Nucleotide sequence of cassette 1 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligo-nucleotides. Oligonucleotide transitions are indicated in the cassette sequence. Lower case letters refer to nucleotide positions outside the open reading frame.

Fig. 1B. Nucleotide sequence of cassette 2 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligo-nucleotides. Oligonucleotide transitions are indicated in the cassette sequence.

Fig. 1C. Nucleotide sequence of cassette 3 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligo-nucleotides. Oligonucleotide transitions are indicated in the cassette sequence. Lower case letters refer to nucleotide positions outside the open reading frame.

Fig. 1D. Nucleotide sequence of the synthetic cutinase gene encoding Fusarium solani pisi pre-pro-cutinase. The cutinase pre-sequence, pro-sequence and mature sequence are indicated. Also the sites used for cloning and the oligonucleotide transitions are indicated. Lower case letters refer to nucleotide positions outside the open reading frame.

Fig. 2. Nucleotide sequence of a synthetic DNA fragment for linking the Fusarium solani pisi pro-cutinase encoding sequence to a sequence encoding a derivative of the E. coli phoA pre-sequence. The ribosome binding site (RBS) and the restriction enzyme sites used for cloning are indicated. Also the amino acid sequences of the encoded phoA signal

sequence and part of the cutinase gene are indicated using the one-letter code.

Fig. 3. Nucleotide sequence of cassette 8, a SacI-BclI fragment which encodes the fusion point of the coding sequences for the invertase pre-sequence and mature Fusarium solani pisi cutinase.

Fig. 4. Plasmid pUR2741 obtained by deletion of a 0.2 kb SalI-NruI from pUR2740, is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with a plant α-galactosidase gene under the control of the yeast gal7 promoter.

Fig. 5. Plasmid pUR7219 is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with the region encoding the mature Fusarium solani pisi cutinase under the control of the yeast gal7 promoter.

Fig. 6. Plasmid pUR2740 is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with a plant α-galactosidase gene under the control of the yeast gal7 promoter.

Fig. 7. Nucleotide sequence of cassettes 5, 6 and 7, comprising different types of connections of the coding sequences of the exlA pre-sequence and mature Fusarium solani pisi cutinase.

Fig. 8. Plasmid pAW14B obtained by insertion of a 5.3 kb SalI fragment of Aspergillus niger var. awamori genomic DNA in the SalI site of pUC19.

Fig. 9. Plasmid pUR7280 obtained by displacing the BspHI-AflII fragment comprising the exlA open reading

frame in pAW14B with a BspHI-AflII fragment comprising the Fusarium solani pisi pre-pro-cutinase coding sequence. Thus, plasmid pUR7280 comprises the Fusarium solani pisi pre-pro-cutinase gene under the control of the A. niger var. awamori promoter and terminator.

Fig. 10. Plasmid pUR7281 obtained by introduction of both the A. nidulans amdS and A. niger var. awamori pyrG selection markers in pUR7280.

Fig. 11. Partial amino acid sequences of the cutinases from Fusarium solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea, showing the location of the residues in the 3-D structure.

Fig. 12. Compatibility of Fusarium solani pisi cutinase and Cutinase variants to a LAS-based detergent composition.

Fig. 13. Compatibility of Fusarium solani pisi cutinase and Cutinase variants to a PAS-based detergent composition.

Fig. 14. Compatibility of Fusarium solani pisi cutinase and Cutinase variants to a high-nonionic detergent composition.

Fig. 15. Compatibility of Fusarium solani pisi cutinase and Cutinase variants to SDS.

Fig. 16. In-the-wash effect for Fusarium solani pisi cutinase and Cutinase variant R17E.

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EXAMPLE 1

Construction of a synthetic gene encoding Fusarium solani pisi pre-pro-cutinase.

A synthetic gene encoding Fusarium solani pisi pre-pro-cutinase was constructed essentially according to the method described in EP-A-407 225 (Unilever). Based on published nucleotide sequences of Fusarium solani pisi genes (Soliday et al. (1984) and WO-A-90/09446, Plant Genetic Systems), a completely synthetic DNA fragment was designed which comprises a region encoding the Fusarium solani pisi pre-pro-cutinase polypeptide. Compared to the nucleotide sequence of the original Fusarium solani pisi gene, this synthetic cutinase gene comprises several nucleotide changes through which restriction enzyme recognition sites were introduced at convenient positions within the gene without affecting the encoded amino acid sequence. The nucleotide sequence of the entire synthetic cutinase gene is presented in Fig. 1D.

Construction of the synthetic cutinase gene was performed by assembly of three separate cassettes starting from synthetic DNA oligonucleotides. Each synthetic DNA cassette is equipped with an EcoRI site at the start and a HindIII site at the end. Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. For the construction of each of the cassettes the procedure outlined below was followed. Equimolar amounts (50 pmol) of the oligonucleotides constituting a given cassette were mixed, phosphorylated at their 5'-end, annealed and ligated according to standard techniques. The resulting mixture of double stranded DNA molecules was cut with EcoRI and HindIII, size-fractionated by agarose gel electrophoresis and recovered from the gel by electro-elution. The resulting synthetic DNA cassette was

ligated with the 2.7 kb EcoRI-HindIII fragment of pUC9 and transformed to Escherichia coli. The EcoRI-HindIII insert of a number of clones was completely sequenced in both directions using suitable oligonucleotide primers to verify the sequence of the synthetic cassettes. Using this procedure pUR7207 (comprising cassette 1, Fig. 1A), pUR7208 (comprising cassette 2, Fig. 1B) and pUR7209 (comprising cassette 3, Fig. 1C) were constructed. Finally, the synthetic cutinase gene was assembled by combining the 2.9 kb EcoRI-ApaI fragment of pUR7207 with the 0.2 kb ApaI-NheI fragment of pUR7208 and the 0.3 kb NheI-HindIII fragment of pUR7209, yielding pUR7210. This plasmid comprises an open reading frame encoding the complete pre-pro-cutinase of Fusarium solani pisi (Fig. 1D).

EXAMPLE 2

Expression of Fusarium solani pisi (pro)cutinase in Escherichia coli.

With the synthetic cutinase gene an expression vector for E. coli was constructed which is functionally similar to the one described in WO-A-90/09446 (Plant Genetic Systems). A construct was designed in which the part of the synthetic gene encoding Fusarium solani pisi pro-cutinase is preceded by proper E. coli expression signals, i.e. (i) an inducible promoter, (ii) a ribosome binding site and (iii) a signal sequence which provides a translational initiation codon and provides information required for the export of the pro-cutinase across the cytoplasmic membrane.

A synthetic linker was designed (see Fig. 2) for fusion of a derivative of the E. coli phoA signal sequence (Michaelis et al., 1983) to the pro-sequence of the synthetic cutinase gene. To optimize cleavage of the signal peptide and secretion of pro-cutinase, the nucleotide sequence of this linker was such that the three C-terminal amino acid residues of the phoA signal sequence (Thr-Lys-Ala) were changed into Ala-Asn-Ala and the N-terminal amino acid residu of the cutinase pro-sequence (Leu 1, see Fig. 1D) was changed into Ala. This construction ensures secretion of cutinase into the

periplasmatic space (see WO-A-90/09446, Plant Genetic Systems).

To obtain such a construct, the 69 bp EcoRI-SpeI fragment comprising the cutinase pre-sequence and part of the pro-sequence was removed from pUR7210 and replaced with the synthetic DNA linker sequence (EcoRI-SpeI fragment) providing the derivative of the E. coli phoA pre-sequence and the alterated N-terminal amino acid residu of the cutinase pro-sequence (Fig. 2). The resulting plasmid was named pUR7250 and was used for the isolation of a 0.7 kb BamHI-HindIII fragment comprising a ribosome binding site and the pro-cutinase encoding region fused to the phoA signal sequence encoding region. This fragment was ligated with the 8.9 kb BamHI-HindIII fragment of pMMB67EH (Fürste et al., 1986) to yield pUR7220. In this plasmid the synthetic gene encoding pro-cutinase is fused to the altered version of the phoA signal sequence and placed under the control of the inducible tac-promoter.

E. coli strain WK6 harboring pUR7220 was grown in 2 litre shakeflasks containing 0.5 litre IXTB medium (Tartof and Hobbs, 1988) consisting of:

0.017 M KH_2PO_4
0.017 M K_2HPO_4
12 g/l Bacto-tryptone
24 g/l Bacto-yeast extract
0.4 % glycerol (v/v)

Cultures were grown overnight at 25°C - 30°C in the presence of 100 µg/ml ampicillin under vigorous shaking (150 rpm) to an OD at 610 nm of 10-12. Then IPTG (isopropyl-ß-D-thiogalactopyranoside) was added to a final concentration of 10 µM and incubation continued for another 12-16 hours. When no further significant increase in the amount of produced lipolytic activity could be observed, as judged by the analysis of samples withdrawn from the cultures, the cells were harvested by centrifugation and resuspended in the original culture volume of buffer containing 20% sucrose at 0°C. The cells were collected by centrifugation and resuspended in the original culture volume of icecold water

causing lysis of the cells through osmotic shock. Cell debris was removed by centrifugation and the cell free extract was acidified to pH 4.8 with acetic acid, left overnight at 4°C and the resulting precipitate was removed. A better than 75% pure cutinase preparation essentially free of endogenous lipases was obtained at this stage by means of ultra-filtration and freeze drying of the cell free extract. Alternatively, cutinase could be purified to homogeneity (i.e. better than 95% pure) by loading the acidified cell free extract onto SP-sephadex, eluting the enzyme with buffer at pH 8.0, passage of the concentatred alkaline solution through a suitable volume of DEAE-cellulose (Whatman DE-52) and direct application of the DEAE flow-through to a Q-sepharose HP (Pharmacia) column. Elution with a salt gradient yielded a homogenous cutinase preparation with a typical overall yield of better than 75%.

EXAMPLE 3

Construction of genes encoding variants of Fusarium solani pisi cutinase.

Using the synthetic gene for Fusarium solani pisi pre-pro-cutinase described in Example 1, variant genes comprising alterations in the encoded amino acid sequence were constructed. For this construction essentially the same approach was followed as described in Example 1 for the construction of the three cassettes constituting the complete synthetic gene. For example, a new version of cassette 1 was assembled using the same oligonucleotides (oligos) as described in Example 1, except for the two oligos which cover the coding triplet for the position which is to be mutated. Instead, two new oligos were used, which comprise the mutant sequence but are otherwise identical to the original oligos which they are replacing.

Example 3A

A gene coding for Fusarium solani pisi cutinase variant R17E was constructed using using a variant of cassette 1 incorporating a variant of CUTI1C IG (containing GAG instead of AGA) and a variant of CUTI1I IG (containing

CTC instead of TCT) instead of CUTI1C IG and CUTI1I IG (see Fig. 1A). The new cassette 1 was cloned and sequenced essentially as described in Example 1 and the about 120 bp EcoRI/NruI DNA fragment comprising the mutation R17E was exchanged for the corresponding fragment in pUR7210, yielding pUR7240 (R17E). The 0.6 kB SpeI-HindIII fragment from this plasmids was used to replace the corresponding fragment in pUR7220, yielding the E. coli expression plasmid pUR7222 (R17E). This E. coli expression plasmid was transformed to E. coli strain WK6. Transformants were grown as outlined in Example 2 and the variant pro-cutinase enzyme was recovered and purified essentially as described in Example 2. Similarly Arg 17 could be replaced by Lys or by Leu.

Example 3B

A gene coding for Fusarium solani pisi cutinase variant R196E was constructed using using a variant of cassette 3 incorporating a variant of CUTI3F MH (containing GAG instead of CGG) and a variant of CUTI3M MH (containing CTC instead of CCG) instead of CUTI3F MH and CUTI3M MH (see Fig. 3A). The new cassette 3 was cloned and sequenced essentially as described in Example 1 and the about 120 bp EcoRI/NruI DNA fragment comprising the mutation R196E was exchanged for the corresponding fragment in pUR7210, yielding pUR7241 (R196E). The 0.6 kB SpeI-HindIII fragment from this plasmids was used to replace the corresponding fragment in pUR7220, yielding the E. coli expression plasmid pUR7225 (R196E). This E. coli expression plasmid was transformed to E. coli strain WK6. Transformants were grown as outlined in Example 2 and the variant pro-cutinase enzyme was recovered and purified essentially as described in Example 2. By the same method Arg 196 was replaced by Lys (R196K), using a variant of CUTI3F MH (containing AAG instead of CGG) and a variant of CUTI3M MH (containing CTT instead of CCG) instead of CUTI3F MH and CUTI3M MH. Similarly, Arg 196 was replaced by Leu (R196L), using a variant of CUTI3F MH (containing CTT instead of CGG) and a variant of CUTI3M MH (containing AAG instead of CCG) instead of CUTI3F MH and CUTI3M MH. The same method was used to replace Arg 196 by Ala (R196A).

Example 3C

A gene coding for Fusarium solani pisi cutinase variant L51A was constructed using using a variant of cassette 1 incorporating a variant of CUTI1F IG (containing GCT instead of CTC) and a variant of CUTI1L IG (containing AGC instead of GAG) instead of CUTI1F IG and CUTI1L IG (see Fig. 1A). The new cassette 1 was cloned and sequenced essentially as described in Example 1 and the about 120 bp EcoRI/NruI DNA fragment comprising the mutation L51A was exchanged for the corresponding fragment in pUR7210, yielding pUR7242 (L51A). The 0.6 kB SpeI-HindIII fragment from this plasmid was used to replace the corresponding fragment in pUR7220, yielding the E. coli expression plasmid pUR7245 (L51A). This E. coli expression plasmid was transformed to E. coli strain WK6. Transformants were grown as outlined in Example 2 and the variant pro-cutinase enzyme was recovered and purified essentially as described in Example 2. Similarly Leu 51 could be replaced by Ser.

Example 3D

Using the cassettes constructed in the examples 3A and 3B, a Cutinase variant with two modifications can be constructed. In example 3A the construction of pUR7240 (R17E) has been described. In example 3B the construction of the EagI/HindlII DNA fragment comprising the mutation R196E has been described. The ApaI/HindlII DNA fragment of pUR7240 (R17E) was replaced by the ApaI/HindlII DNA fragment of pUR7241, yielding pUR7243 (R17E+R196E). The 0.6 kB SpeI-HindIII fragment from this plasmid was used to replace the corresponding fragment in pUR7220, yielding the E. coli expression plasmid pUR7226 (R17E+R196E). This E. coli expression plasmid was used to transform to E. coli strain WK6. Transformants were grown as outlined in Example 2 and the variant pro-cutinase enzyme was recovered and purified essentially as described in Example 2.

Example 3E

Using the cassettes constructed in the examples 3A and 3C, a Cutinase variant with two modifications can be constructed. In example 3A the construction of pUR7240 (R17E)

has been described. In example 3C the construction of the DNA fragment comprising the mutation L51A has been described. The BclI/ApaI fragment of pUR7242 was exchanged for the corresponding fragment in pUR7240, yielding pUR7244 (R17E+L51A). The 0.6 kB SpeI-HindIII fragment from this plasmids was used to replace the corresponding fragment in pUR7220, yielding the E. coli expression plasmid pUR7246 (R17E+L51A). This E. coli expression plasmid was used to transform E. coli strain WK6. Transformants were grown as outlined in Example 2 and the variant pro-cutinase enzyme was recovered and purified essentially as described in Example 2.

EXAMPLE 4

Expression of Fusarium solani pisi cutinase in Saccharomyces cerevisiae.

For the expression of the synthetic Fusarium solani pisi cutinase gene in Saccharomyces cerevisiae an expression vector was constructed in which a synthetic gene encoding the mature cutinase is preceded by the pre-sequence of S. cerevisiae invertase (Taussig and Carlsson, 1983) and the strong, inducible gal7 promoter (Nogi and Fukasawa, 1983). To prepare the synthetic cutinase gene for such a fusion, an adaptor fragment was synthetized in which the coding sequence for the invertase pre-sequence is fused to the sequence encoding the N-terminus of mature cutinase. This fragment was assembled as an EcoRI-HindIII cassette in pUC9 essentially as described in Example 1 (cassette 8, see Fig. 3), yielding pUR7217. Plasmids pUR7210 and pUR7217 were transformed to E. coli JM110 (a strain lacking the dam methylase activity) and the 2.8 kb BclI-HindIII fragment of pUR7217 was ligated with the 0.6 kb BclI-HindIII fragment of pUR7210, yielding pUR7218 in which the nucleotide sequence coding for the mature cutinase polypeptide is fused with part of the S. cerevisiae invertase pre-sequence coding region.

The expression vector pUR2741 (see Fig. 4) was derived from pUR2740 (Verbakel, 1991, see Fig. 6) by isolation of the 8.9 kb NruI-SalI fragment of pUR2740, filling in the SalI protruding end with Klenow polymerase,

and recircularization of the fragment. The 7.3 kb SacI-HindIII fragment of pUR2741 was ligated with the 0.7 kb SacI-HindIII fragment of pUR7218, yielding pUR7219 (see Fig. 5). Optionally, a S. cerevisiae polII terminator can be placed behind the cutinase gene, in the HindIII site, which turned out not to be essential for efficient expression of the cutinase gene. The E. coli-S. cerevisiae shuttle plasmid pUR7219 contains a origin for replication in S. cerevisiae strains harboring the 2μ plasmid (cir^+ strains), a promoter-deficient version of the S. cerevisiae Leu2 gene permitting selection of high copy number transformants in S. cerevisiae $leu2^-$ strains, and the synthetic gene encoding the mature part of Fusarium solani pisi cutinase operably linked to the S. cerevisiae invertase pre-sequence under the regulation of the strong, inducible S. cerevisiae gal7 promoter.

S. cerevisiae strain SU50 (a, cir^0, leu2, his4, can1), which is identical to strain YT6-2-1L (Erhart and Hollenberg, 1981), was co-transformed with an equimolar mixture of the 2μ S. cerevisiae plasmid and pUR7219 using a standard protocol for electroporation of yeast cells. Transformants were selected for leucine prototrophy and total DNA was isolated from a number of transformants. All transformants appeared to contain both the 2μ plasmid and pUR7219, exemplifying that the promoter-deficient version of the leu2 gene contained on pUR7219 can only functionally complement leu2 deficient strains when present in high copy numbers due to the simultaneous presence of the 2μ yeast plasmid. One of the transformants was cured for the pUR7219 plasmid by cultivation on complete medium for more than 40 generations followed by replica-plating on selective and complete solid media, yielding S. cerevisiae strain SU51 (a, cir^+, leu2, his4, can1).

S. cerevisiae strain SU51 harboring pUR7219 was grown in 1 litre shakeflasks containing 0.2 litre MM medium consisting of:

- yeast nitrogen base (YNB) without amino acids	6.7	g/l
- histidine	20	mg/l
- glucose	20	g/l

Cultures were grown overnight at 30°C under vigorous shaking (150 rpm) to an OD at 610 nm of 2-4. Cells were collected by centrifugation and resuspended in 1 litre of YPGAL medium consisting of:

- yeast extract 10 g/l
- bacto peptone 20 g/l
- galactose 50 g/l

in 2 litre shake flasks and incubation continued for another 12-16 hours. At regular intervals samples were withdrawn from the culture and centrifugated to remove biomass. The supernatant was analyzed for cutinase activity by a titrimatic assay using olive oil as a substrate. For each sample between 100 and 200 µl of filtrate was added to a stirred mixture of 5.0 ml lipase substrate (Sigma, containing olive oil as a substrate for the lipase) and 25.0 ml of buffer (5 mM Tris-HCl pH 9.0, 40 mM NaCl, 20 mM $CaCl_2$). The assay was carried out at 30°C and the release of fatty acids was measured by automated titration with 0.05 M NaOH to pH 9.0 using a Mettler DL25 titrator. A curve of the amount of titrant against time was obtained. The amount of lipase activity contained in the sample was calculated from the maximum slope of this curve. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of fatty acid from olive oil in one minute under the conditions specified above. Such determinations are known to those skilled in the art.

When the production of cutinase activity did no longer increase, cells were removed by centrifugation and the cell free extract was acidified to pH 4.8 with acetic acid and cutinase was recovered as described in Example 1.

EXAMPLE 5

Expression of variants of Fusarium solani pisi cutinase in S. cerevisiae.

The 0.5 kb ApaI-HindIII fragment of pUR7241 (R196E) was used to replace the analogous fragment of pUR7218, yielding pUR7229 (R196E), in which the gene comprising the mutation is operably fused to the sequence encoding the S.

cerevisiae signal sequence. The 7.0 kb SacI-HindIII fragment of pUR2741 was ligated with the 0.7 kb SacI-HindIII fragment of pUR7229 (R196E), yielding pUR7235 (R196E). This plasmid was used to transform to S. cerevisiae strain SU51. The resulting transformants were incubated as described in Example 4 and the variant enzyme produced was recovered from the culture broth as described in Examples 4 and 1.

EXAMPLE 6

Expression of Fusarium solani pisi cutinase in Aspergilli.

For the expression of the synthetic Fusarium solani pisi cutinase gene in Aspergillus niger var. awamori an expression vector was constructed in which the synthetic gene encoding Fusarium solani pisi pre-pro-cutinase was placed under the control of the A. niger var. awamori strong, inducible exlA promoter (Maat et al.,1992, de Graaff et al., 1992).

The pre-pro-cutinase expression plasmid (pUR7280) was constructed starting from plasmid pAW14B, which was deposited in an E. coli strain JM109 with the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, under N° CBS 237.90 on 31st May 1990, and contains a ca. 5.3 kb SalI fragment on which the 0.7 kb endoxylanase II (exlA) gene is located, together with 2.5 kb of 5'-flanking sequences and 2.0 kb of 3'-flanking sequences (Fig.8). In pAW14B the exlA coding region was replaced by the pre-pro-cutinase coding region. A BspHI site (5'-TCATGA-3') comprising the first codon (ATG) of the exlA gene and an AflII site (5'-CTTAAG-3'), comprising the stopcodon (TAA) of the exlA gene facilitated the construction of pUR7280.

The construction was carried out as follows: pAW14B (7.9 kb) was cut partially with BspHI and the linearized plasmid (7.9 kb) was isolated from an agarose gel. Subsequently the isolated 7.9 kb fragment was cut with BsmI, which cuts a few nucleotides downstream of the BspHI site of interest, to remove plasmids linearized at other BspHI sites. The fragments were separated on an agarose gel and the 7.9 kb BspHI-BsmI fragment was isolated. This was partially cut with

AflII and the resulting 7.2 kb BspHI-AflII fragment was isolated.

The 0.7 kb BspHI-AflII fragment of pUR7210 comprising the entire open reading frame coding for Fusarium solani pisi pre-pro-cutinase was ligated with the 7.2 kb BspHI-AflII fragment of pAW14B, yielding pUR7280. The constructed vector (pUR7280) can subsequently transferred to moulds (for example Aspergillus niger, Aspergillus niger var. awamori, etc) by means of conventional co-transformation techniques and the pre-pro-cutinase gene can then be expressed via induction of the endoxylanaseII promoter. The constructed rDNA vector can also be provided with conventional selection markers (e.g. amdS or pyrG, hygromycin etc.) and moulds can be transformed with the resulting rDNA vector to produce the desired protein. As an example, the amdS and pyrG selection markers were introduced in the expression vector, yielding pUR7281 (Fig. 10). For this purpose a NotI site was created by converting the EcoRI site (present 1.2 kb upstream of the ATG codon of the pre-pro-cutinase gene) into a NotI site using a synthetic oligonucleotide (5'-AATTGCGGCCGC-3'), yielding pUR7282. Suitable DNA fragment comprising the entire A. nidulans amdS gene and the A. niger var. awamori pyrG gene together with their own promoters and terminators were equiped with flanking NotI sites and introduced in the NotI site of pUR7282, yielding pUR7281 (Fig. 10).

As an alternative approach for the expression of the synthetic Fusarium solani pisi cutinase gene in Aspergillus niger var. awamori, expression vectors were constructed in which a synthetic gene encoding the mature cutinase is not preceded by its own pre-pro-sequence, but by the pre-sequence of A. niger var. awamori exlA.

To prepare the synthetic cutinase gene for such fusions, several adaptor fragments were synthetized in which the coding sequence for the exlA pre-sequence is connected to the sequence encoding the N-terminus of mature cutinase in different ways. In cassette 5 this connection is made by fusing the exlA pre-sequence to the pro-sequence of cutinase.

In cassette 6 the exlA pre-sequence is fused with the N-terminal residu of mature cutinase. Cassette 7 is identical with cassette 6, but here the N-terminal residue of the encoded mature cutinase polypeptide has been changed from the original Glycine into a Serine residue in order to better fit the requirements for cleavage of the signal peptide. Cassettes 5, 6 and 7 were assembled from synthetic oligonucleotides essentially as described in Example 1 (see Fig. 7). Cassette 5 was used to displace the 0.1 kb EcoRI-SpeI fragment of pUR7210, yielding pUR7287. Cassettes 6 and 7 were used to displace the 0.1 kb EcoRI-BclI fragment of pUR7210, yielding pUR7288 and pUR7289, respectively. For each of the plasmids pUR7287, pUR7288 and pUR7289 the 0.7 kb BspHI-AflII fragment was ligated with the 7.2 kb BspHI-AflII fragment of pAW14B, yielding pUR7290, pUR7291 and pUR7292, respectively.

The constructed rDNA vectors subsequently were transferred to moulds (Aspergillus niger, Aspergillus niger var. awamori) by means of conventional co-transformation techniques and the pre-(pro)-cutinase gene were expressed via induction of the endoxylanaseII promoter. The constructed rDNA vectors can also be provided with conventional selection markers (e.g. amdS or pyrG, hygromycin) and the mould can be transformed with the resulting rDNA vector to produce the desired protein, as illustrated in this example for pUR7280 (see above).

Aspergillus strains transformed with either of the expression vectors pUR7280, pUR7281, pUR7290, pUR7291, pUR7292 (containing the Fusarium solani pisi mature cutinase encoding region with or without the corresponding pro-sequence and either the cutinase signal sequence or the exlA signal sequence under the control of A. niger var. awamori exlA promoter and terminator) were grown under the following conditions: multiple 1 litre shake flasks with 400 ml synthetic media (pH 6.5) were inoculated with spores (final concentration: 10E6/ml). The medium had the following composition (AW Medium):

sucrose	10 g/l
$NaNO_3$	6.0 g/l
KCl	0.52 g/l
KH_2PO_4	1.52 g/l
$MgSO_4 \cdot 7H_2O$	0.49 g/l
Yeast extract	1.0 g/l
$ZnSO_4 \cdot 7H_2O$	22 mg/l
H_3BO_3	11 mg/l
$MnCl_2 \cdot 4H_2O$	5 mg/l
$FeSO_4 \cdot 7H_2O$	5 mg/l
$CaCl_2 \cdot 6H_2O$	1.7 mg/l
$CuSO_4 \cdot 5H_2O$	1.6 mg/l
$NaH_2MoO_4 \cdot 2H_2O$	1.5 mg/l
Na_2EDTA	50 mg/l

Incubation took place at 30°C, 200 rpm for 24 hours in a Mk X incubator shaker. After growth cells were collected by filtration (0.45 μm filter), washed twice with AW Medium without sucrose and yeast extract (salt solution), resuspended in 50 ml salt solution and transferred to 300 ml shake flasks containing 50 ml salt solution to which xylose has been added to a final concentration of 10 g/l (induction medium). Incubation under the same conditions as described above was continued overnight. The resulting cultures were filtered over miracloth to remove biomass and cutinase was recovered essentially as described in Example 2.

EXAMPLE 7

Expression of variants of Fusarium solani pisi cutinase in Aspergilli.

By following essentially the route outlined in Example 6, but now using plasmid pUR7240 (R17E) or pUR7241 (R196E) or pUR7242 (L51A) instead of pUR7210 for the construction of fungal expression vectors, variants of Fusarium solani pisi cutinase comprising the above mentioned mutations were produced in Aspergillus niger var. awamori.

EXAMPLE 8

Identification and isolation of genes related to the Fusarium solani pisi cutinase gene.

Genes encoding cutinases with a varying degree of homology with Fusarium solani pisi cutinase were isolated from different fungi. Fungal cultures were grown in 500 ml shakeflasks containing 200 ml of the medium described by Hankin and Kolattukudy (1968) supplemented with 0.25% glucose and incubated for 4 days at 28°C in a Mk X incubator shaker (100 rpm). At this time the glucose had been consumed and cutinase production was induced by the addition of cutin hydrolysate essentially as described by Ettinger et al. (1987). At regular intervals samples were withdrawn from the culture and analyzed for the presence of lipolytic activity according to standard techniques (see Example 4). Normally, about two days after induction lipolytic activity could be demonstrated and at that time the cells were harvested by filtration using standard techniques. The mycelia were washed, frozen in liquid nitrogen and lyophilized according to standard techniques. Total cellular RNA preparations were isolated using the guanidinium thiocyanate method and purified by cesium chloride density gradient centrifugation, essentially as described by Sambrook et al. (1989). PolyA(+) mRNA fractions were isolated using a polyATtract mRNA isolation kit (Promga). The polyA(+) mRNA fractions were used in a Northern hybridization analysis using a cDNA fragment from the Fusarium solani pisi cutinase gene as a probe according to standard techniques, to verify the expression of cutinase-related genes. Preparations of mRNA comprising material capable of hybridizing with the probe were used for the synthesis of cDNA using a ZAP cDNA synthesis kit (Stratagene, La Jolla) according to the instructions of the supplier, yielding cDNA fragments with an XhoI cohesive end flanking the poly-A region and an EcoRI adaptor at the other end. The obtained cDNA fragments were used for the construction of expression libraries by directional cloning in the sense orientation in lambda ZAPII vectors (Stratagene, La Jolla), allowing expression of ß-galactosidase fusion

proteins (Huse et al.,1988). These libraries were screened using antiserum raised against Fusarium solani pisi cutinase.

Alternatively, the synthesized cDNA fractions were subjected to PCR-screening using cutinase specific primers (see table 2). These primers were derived from comparison of the amino acid sequence of several fungal Cutinase genes (Ettinger et al., 1987). The conditions for the PCR reaction were optimized for each set of primers, using cDNA from Fusarium solani pisi cutinase as a control. For those preparations of cDNA with which a specific PCR fragment could be generated with a length that is similar to (or greater than) the length of the PCR fragment generated with the cDNA from Fusarium solani pisi cutinase under identical conditions, the PCR fragment was purified by gel electroforesis and isolated from the gel.

As an alternative approach, the PCR screening techique using Cutinase specific primers was also applied directly to genomic DNA of some fungal strains, using genomic DNA of Fusarium solani pisi as a positive control. For those preparations of fungal genomic DNA with which a specific PCR fragment could be generated with a length that is similar to (or greater than) the length of the PCR fragment generated with the cDNA from Fusarium solani pisi cutinase under identical conditions, the PCR fragment was purified by gel electroforesis and isolated from the gel.

For strains which scored positive in either the expression library approach or the PCR screening approach (either with cDNA or genomic DNA) as well as a number of other strains, high molecular weight genomic DNA was isolated. Strains were grown essentially as described by Ettinger et al. (1987), and genomic DNA was isolated as described by de Graaff et al. (1988). Genomic DNA was digested with various restriction enzymes and analyzed by Southern hybridization using either the analogous cDNA insert (expression library approach) or the PCR fragment (PCR screening approach) or the Fusarium solani pisi cutinase gene (other strains) as a probe, and a physical map of the cutinase genes was constructed. An appropriate digest of

genomic DNA was size-fractionated by gel electroforesis and fragments of the appropriate size were isolated from the gel and subcloned in pUC19. These genomic libraries were screened with the corresponding cDNA insert (expression library approach) or the PCR fragment (PCR screening approach), yielding clones comprising the genomic copy of the cutinase genes. These genes were sequenced in both directions. Introns were identified by sequencing the corresponding cDNA or by comparison with other Cutinase sequences (Ettinger et al., 1987). The N-terminal end of the mature cutinase polypeptide was also deduced from such a comparison. Using standard PCR techniques, the introns were removed, a HindIII site was engineered immediately downstream of the open reading frames and the coding sequence for the pre-sequence of the Saccharomyces cerevisiae invertase gene (preceded by a SacI site, compare cassette 8, Fig. 3) was fused to the sequences encoding the N-terminus of the mature cutinases. The obtained SacI-HindIII fragments comprising the cutinase genes operably linked to the sequence encoding the S. cerevisiae invertase pre-sequence were ligated with the 7.3 kb SacI-HindIII fragment of pUR7241 (see Fig. 4) and transformed to S. cerevisiae strain SU51. The fungal cutinases were expressed and recovered from the culture broth essentially as described in Example 4.

EXAMPLE 9

The compatibility of Fusarium solani pisi Cutinase variants R17E, R196E and R17E+R196E to various anionic surfactants.

The compatibility of Fusarium solani pisi cutinase and of the Fusarium solani pisi cutinase variants R17E, R196E and R17E+R196E to various anionic surfactants was tested as follows. Solutions of the enzyme in various detergent products were prepared. The solutions were incubated at 40°C and at intervals samples were taken. Then the enzyme activity was determined following the assay described in Example 4. The following detergent products A-C were used:

Product A

compound	weight %
Na-Linear alkyl benzene sulphonate	11.7
Nonionic surfactant 7EO	5.8
Nonionic surfactant 3EO	3.2
Zeolite	38.8
Sokolan CP7	4.8
Sodium CMC	0.8
Sodium carbonate	13.9
Sodium perborate	8.0
TAED	5.4
Sodium silicate	2.5

Product B

compound	weight %
Sodium Primary Alkyl Sulphate	6.5
Nonionic surfactant 7EO	6.5
Nonionic surfactant 3EO	8.3
Soap	2.3
Zeolite	38.0
Sodium carbonate	15.9
Sodium perborate	8.0
TAED	5.4
Sodium silicate	2.5

Product C

compound	weight %
Na-Linear alkyl benzene sulphonate	6.9
Nonionic surfactant	10.0
Soap	2.0
Zeolite	27.0
Sodium carbonate	10.2

The results for the compositions A-C are given in Figure 12-14. It follows that, in particular in the anionic-rich composition A, the Cutinase variants are more stable than wildtype Fusarium solani pisi cutinase.

EXAMPLE 10

The compatibility of Fusarium solani pisi Cutinase variants R196K and R196L to Sodium Dodecyl Sulphate (SDS).

The compatibility of Fusarium solani pisi cutinase and of the Fusarium solani pisi cutinase variants R196K and R196L to Sodium Dodecyl Sulphate (SDS) was tested as follows. Solutions of the enzymes in 0.4 mM SDS and 10 mM Tris at 0°FH were prepared. The solutions were incubated at 40°C and at intervals samples were taken and the residual enzyme activity was determined following the assay described in Example 4. The results are shown in Figure 15. It can be seen that both Cutinase variants are more stable to the anionic surfactant Sodium Dodecyl Sulphate (SDS) than wildtype Fusarium solani pisi cutinase.

EXAMPLE 11

Determining the In-the-wash activity of Fusarium solani pisi Cutinase variant R17E.

Test cloths made of woven polyester/cotton were soiled with pure olive oil. Each tests cloth was then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The bottles were agitated in a Miele TMT washing

machine filled with water and using a normal 40°C main wash programme. The wash liquor consisted of 2 grams per litre (at 27°FH) of washing powders A and B of Example 9.

The results are shown in Figure 16. The enhancement of the in-the-wash performance (oily soil removal) of Cutinase varaiant R17E relative to wild-type Fusarium solani pisi cutinase under various wash conditions is evident. For comparison, the same experiments were also carried out with Lipolase (TM). Under all conditions, the Cutinase variant R17E was superior.

CLAIMS

1. A Cutinase variant of a parent Cutinase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved.

2. A Cutinase variant according to Claim 1, in which the compatibility to anionic surfactants has been improved by reducing the binding of anionic surfactants to the enzyme.

3. A Cutinase variant according to any one of the preceding Claims, in which the binding of anionic surfactants to the enzyme has been reduced by reducing the electrostatic interaction between the anionic surfactant and the enzyme.

4. A Cutinase variant according to any one of Claims 1-3, wherein the electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by lysine residues.

5. A Cutinase variant according to any one of Claims 1-3, wherein the electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by uncharged amino acid residues.

6. A Cutinase variant according to any one of Claims 1-3, wherein the electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by negatively charged amino acid residues.

7. A Cutinase variant according to any one of Claims 1-3, wherein the binding of the anionic surfactant and the enzyme is reduced by replacing one or more amino acid residues which are located in a hydrophobic patch capable of binding the apolair tail of the anionic surfactant, by less hydrophobic amino acid residues.

8. A Cutinase variant according to Claim 7, in which the less hydrophobic amino acid residues are selected from the group consisting of glycine, serine, alanine, aspartic acid and threonine.

9. A Cutinase variant according to any of the preceding Claims, wherein the parent Cutinase is an eukaryotic Cutinase.

10. A Cutinase variant according to any one of the preceding Claims, in which the parent enzyme is a Cutinase which is immunologically cross-reacting with antibodies raised against the cutinase from Fusarium solani pisi.

11. A Cutinase variant according to any one of the preceding Claims, encoded by genes that have extensive homology to the 5'- and/or 3' ends of to the genes encoding cutinase from Fusarium solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes, Magnaporthe grisiea and/or to conserved sequences in Cutinases.

12. A Cutinase variant according to any one of the preceding Claims, in which the modified residues are located at one or more of the following positions in the amino acid sequence of the Fusarium solani pisi cutinase, or the corresponding amino acids of a different Cutinase: 17, 51, 78, 80, 88, 96, 156, 195 and 196.

13. A Cutinase variant according to any one of the preceding Claims, whereby the modified residues are located in the hydrophobic patch around amino acids 51 and 195 of the

Fusarium solani pisi cutinase, or the corresponding amino acids of a different Cutinase.

14. A Cutinase variant according to any one of the preceding Claims, which is a variant of Magnoporthe grisiea cutinase and comprises one or more of the following mutations: A80D, A88E, R156L.

15. A process for producing a Cutinase variant according to any one of the preceding Claims, which comprises the steps of fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the Cutinase variant, making a preparation of the Cutinase variant by separating the Cutinase variant produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the Cutinase either from said broth or from said cells by physical or chemical concentration or purification methods.

16. A rDNA modified micro-organism which has been transformed by a rDNA vector carrying a gene encoding a Cutinase variant according to any of Claims 1 to 14 and which is thereby able to express said Cutinase variant.

17. A rDNA modified micro-organism according to Claim 16 carrying a gene encoding a Cutinase variant that is introduced into the micro-organism by fusion at its 5'-end to a gene fragment encoding a (modified) pre-sequence functional as a signal- or secretion-sequence for the host organism.

18. A rDNA modified micro-organism according to Claims 16 or 17, wherein the host organism is a eukaryote, for example a yeast of the genus Saccharomyces or Kluyveromyces or the genus Hansenula, or a fungus of the genus Aspergillus.

19. An rDNA modified micro-organism according to Claims 16 to 18, carrying a recombinant DNA vector coding for a Cutinase variant according to any of Claims 1 - 14, said micro-organism having being made an auxotrophic mutant by gene replacement of the gene coding for the auxotrophic marker in one of its ancestor cells.

20. A polynucleotide having a base sequence that encodes the mature Cutinase variant according to any one of Claims 1 - 14 or a functional equivalent or a mutant thereof, in which polynucleotide the final translated codon is followed by a stop codon and optionally having nucleotide sequences coding for the pre-sequence of this Cutinase directly upstream of the nucleotide sequences coding for the mature enzyme.

21. A polynucleotide having a base sequence encoding a Cutinase variant according to any of Claims 1 - 14, in which polynucleotide the final translated codon is followed by a stop codon and optionally having a nucleotide sequence coding for at least a part of the corresponding presequence, and/or a signal- or secretion-sequence suitable for a selected host organism, directly upstream of the nucleotide sequence coding for the mature enzyme.

22. A polynucleotide having a base sequence that encodes the mature Cutinase variant according to any one of Claims 1 - 14, or a functional equivalent or mutant thereof, in which the Cutinase-variant encoding nucleotide sequence derived from the organism of origin has been modified in such a way that at least one codon, and preferably as many codons as possible, have been made the subject of 'silent' mutations to form codons encoding equivalent amino acid residues and being codons preferred by a new host as specified in one of Claims 16 to 19, thereby to provide in use within the cells of such host a messenger-RNA for the introduced gene of improved stability.

23. A polynucleotide according to any one of Claims 20 to 22, in which upstream of the nucleotide sequences coding for the pro-or mature Cutinase variant, there is located a nucleotide sequence that codes for a signal or secretion sequence suitable for a host as specified in any one of Claims 16 to 19.

23. A recombinant DNA vector able to direct the expression of a nucleotide sequence encoding a Cutinase variant gene, comprising the following components:
(a) Double-stranded (ds) DNA coding for the mature Cutinase variant or precutinase or a corresponding precutinase in which at least part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell), provided that where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front, and provided also that the part of the gene to be translated ends with an appropriate stop codon or has such codon added;
(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the Cutinase variant (component (a));
(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the Cutinase variant (component (a);
(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,
(d2) an origin of replication suitable for the selected host;
(e1) Optionally a (auxotrophic) selection marker;
(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the Cutinase variant in the host selected.

25. A recombinant DNA vector according to Claim 24, also carrying, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the Cutinase.

26. A recombinant DNA vector according to any one of Claims 24 to 25, carrying an auxotrophic marker consisting of a coding region of the auxotrophic marker and a defective promotor region.

27. An enzymatic detergent composition comprising a Cutinase variant according to any one of Claims 1 to 14.

*****

Fig.1A.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE I

CODE	Length	5' <--- sequence ---> 3'
CUTI1A IG	(44)	aat tcg agc tca tcA TGA AAT TCT TCG CGT TAA CCA CAC TTC TC
CUTI1B IG	(39)	GCC GCC ACG GCT TCG GCT CTG CCT ACT AGT AAC CCT GCT
CUTI1C IG	(42)	CAG GAG CTT GAG GCG CGC CAG CTT GGT AGA ACA ACT CGC GAC
CUTI1D IG	(39)	GAT CTG ATC AAC GGC AAT AGC GCT TCC TGC GCC GAT GTC
CUTI1E IG	(33)	ATC TTC ATT TAT GCT CGA GGT TCA ACA GAG ACG
CUTI1F IG	(28)	GGC AAC TTG GGA ACT CTC GGG CCC AGC A
CUTI1G IG	(31)	GGT TAA CGC GAA GAA TTT CAT gat gag ctc g
CUTI1H IG	(39)	ACT AGT AGG CAG AGC CGA AGC CGT GGC GGC GAG AAG TGT
CUTI1I IG	(42)	TGT TCT ACC AAG CTG GCG CGC CTC AAG CTC CTG AGC AGG GTT
CUTI1J IG	(39)	GCA GGA AGC GCT ATT GCC GTT GAT CAG ATC GTC GCG AGT
CUTI1K IG	(33)	TGA ACC TCG AGC ATA AAT GAA GAT GAC ATC GGC
CUTI1L IG	(41)	AGC TTG CTG GGC CCG AGA GTT CCC AAG TTG CCC GTC TCT GT

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        10          20          30          40          50          60
               aattcg agctcatcAT GAAATTCTTC GCGTTAACCA CACTTCTCGC
                   gc tcgagtagTA CTTTAAGAAG CGCAATTGGT GTGAAGAGCG

        70          80          90          100         110         120
CGCCACGGCT TCGGCTCTGC CTACTAGTAA CCCTGCTCAG GAGCTTGAGG CGCGCCAGCT
GCGGTGCCGA AGCCGAGACG GATGATCATT GGGACGAGTC CTCGAACTCC GCGCGGTCGA

       130         140         150         160         170         180
TGGTAGAACA ACTCGCGACG ATCTGATCAA CGGCAATAGC GCTTCCTGCG CCGATGTCAT
ACCATCTTGT TGAGCGCTGC TAGACTAGTT GCCGTTATCG CGAAGGACGC GGCTACAGTA

       190         200         210         220         230         240
CTTCATTTAT GCTCGAGGTT CAACAGAGAC GGGCAACTTG GGAACTCTCG GGCCCAGCA
GAAGTAAATA CGAGCTCCAA GTTGTCTCTG CCCGTTGAAC CCTTGAGAGC CCGGGTCGTT

       250

GGA
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Fig.1B.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 2

CODE	Length	5' <--- sequence ---> 3'
CUTI2A MH	(40)	AAT TCT CGG GCC CAG CAT TGC CTC CAA CCT TGA GTC CGC C
CUTI2B MH	(36)	TTC GGC AAG GAC GGT GTC TGG ATT CAG GGC GTT GGC
CUTI2C MH	(36)	GGT GCC TAC CGA GCC ACC CTA GGA GAC AAT GCT CTC
CUTI2D MH	(39)	CCG CGG GGA ACC TCT AGC GCC GCA ATC AGG GAG ATG CTA
CUTI2E MH	(45)	GGC CTC TTC CAG CAG GCC AAC ACC AAG TGC CCT GAC GCG ACT TTG
CUTI2F MH	(46)	ATC GCC GGT GGC TAC AGC CAG GGT GCT GCA CTT GCA GCC GCT AGC A
CUTI2G MH	(45)	CTT GCC GAA GGC GGA CTC AAG GTT GGA GGC AAT GCT GGG CCC GAG
CUTI2H MH	(36)	GTA GGC ACC GCC AAC GCC CTG AAT CCA GAC ACC GTC
CUTI2I MH	(36)	TCC CCG CGG GAG AGC ATT GTC TCC TAG GGT GGC TCG
CUTI2J MH	(39)	GAA GAG GCC TAG CAT CTC CCT GAT TGC GGC GCT AGA GGT
CUTI2K MH	(45)	ACC GGC GAT CAA AGT CGC GTC AGG GCA CTT GGT GTT GGC CTG CTG
CUTI2L MH	(41)	AGC TTG CTA GCG GCT GCA AGT GCA GCA CCC TGG CTG TAG CC

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      10          20          30          40          50          60
  AATTCTC GGGCCCAGCA TTGCCTCCAA CCTTGAGTCC GCCTTCGGCA AGGACGGTGT
      GAG CCCGGGTCGT AACGGAGGTT GGAACTCAGG CGGAAGCCGT TCCTGCCACA

      70          80          90          100         110         120
CTGGATTCAG GGCGTTGGCG GTGCCTACCG AGCCACCCTA GGAGACAATG CTCTCCGCG
GACCTAAGTC CCGCAACCGC CACGGATGGC TCGGTGGGAT CCTCTGTTAC GAGAGGGCGC

      130         140         150         160         170         180
GGGAACCTCT AGCGCCGCAA TCAGGGAGAT GCTAGGCCTC TTCCAGCAGG CCAACACCAA
CCCTTGGAGA TCGCGGCGTT AGTCCCTCTA CGATCCGGAG AAGGTCGTCC GGTTGTGGTT

      190         200         210         220         230         240
GTGCCCTGAC GCGACTTTGA TCGCCGGTGG CTACAGCCAG GGTGCTGCAC TTGCAGCCGC
CACGGGACTG CGCTGAAACT AGCGGCCACC GATGTCGGTC CCACGACGTG AACGTCGGCG

      250
TAGCA
ATCGTTCGA
```

Fig.1C.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 3

CODE	Length	5' <--- sequence ---> 3'
CUTI3A	MH (43)	AAT TCC CGC TAG CAT CGA GGA CCT CGA CTC GGC CAT TCG TGA C
CUTI3B	MH (45)	AAG ATC GCC GGT ACC GTT CTG TTC GGC TAC ACC AAG AAC CTA CAG
CUTI3C1	MH (42)	AAT CGC GGC CGA ATC CCC AAC TAC CCT GCC GAC AGG ACC AAG
CUTI3D	MH (42)	GTC TTC TGC AAT ACA GGA GAT CTC GTT TGT ACT GGT AGC TTG
CUTI3E	MH (39)	ATC GTT GCT GCA CCT CAC TTG GCA TAT GGT CCT GAT GCC
CUTI3F	MH (33)	CGG GGA CCT GCC CCT GAG TTC CTC ATC GAG AAG
CUTI3G1	MH (32)	GTT CGG GCT GTC CGT GGT TCT GCT TGA gct ta
CUTI3H	MH (30)	GGC CGA GTC GAG GTC CTC GAT GCT AGC GGG
CUTI3I	MH (45)	CTT GGT GTA GCC GAA CAG AAC GGT ACC GGC GAT CTT GTC ACG AAT
CUTI3J1	MH (42)	GTC GGC AGG GTA GTT GGG GAT TCG GCC GCG ATT CTG TAG GTT
CUTI3K	MH (42)	AGT ACA AAC GAG ATC TCC TGT ATT GCA GAA GAC CTT GGT CCT
CUTI3L	MH (39)	ACC ATA TGC CAA GTG AGG TGC AGC AAC GAT CAA GCT ACC
CUTI3M	MH (33)	GAG GAA CTC AGG GGC AGG TCC CCG GGC ATC AGG
CUTI3N1	MH (45)	agc tta agc TCA AGC AGA ACC ACG GAC AGC CCG AAC CTT CTC GAT

```
        10          20          30          40          50          60
 AATTCCCGC TAGCATCGAG GACCTCGACT CGGCCATTCG TGACAAGATC GCCGGTACCG
    GGGCG ATCGTAGCTC CTGGAGCTGA GCCGGTAAGC ACTGTTCTAG CGGCCATGGC

        70          80          90          100         110         120
TTCTGTTCGG CTACACCAAG AACCTACAGA ATCGCGGCCG AATCCCCAAC TACCCTGCCG
AAGACAAGCC GATGTGGTTC TTGGATGTCT TAGCGCCGGC TTAGGGGTTG ATGGGACGGC

        130         140         150         160         170         180
ACAGGACCAA GGTCTTCTGC AATACAGGAG ATCTCGTTTG TACTGGTAGC TTGATCGTTG
TGTCCTGGTT CCAGAAGACG TTATGTCCTC TAGAGCAAAC ATGACCATCG AACTAGCAAC

        190         200         210         220         230         240
CTGCACCTCA CTTGGCATAT GGTCCTGATG CCCGGGGACC TGCCCCTGAG TTCCTCATCG
GACGTGGAGT GAACCGTATA CCAGGACTAC GGGCCCCTGG ACGGGGACTC AAGGAGTAGC

        250         260         270         280
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TCTTCCAAGC CCGACAGGCA CCAAGACGAA CTcgaattcg a
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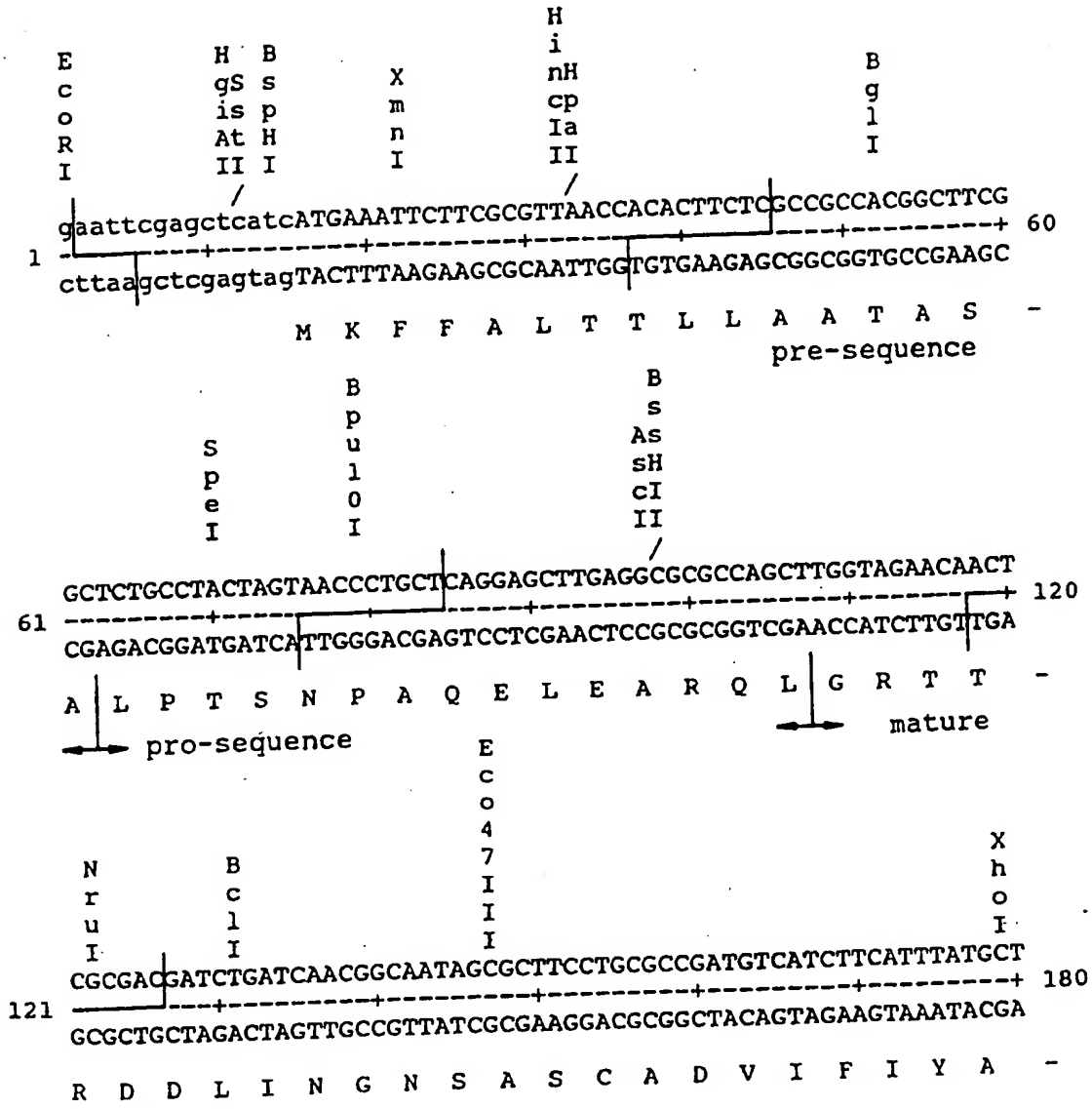
Fig.1D(1 of 4).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BglI
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1
60
M K F F A L T T L L A A T A S
pre-sequence
SpeI
BpuI0I
BsAssHcIII
GCTCTGCCTACTAGTAACCCTGCTCAGGAGCTTGAGGCGCGCCAGCTTGGTAGAACAACT
CGAGACGGATGATCATTGGGACGAGTCCTCGAACTCCGCGCGGTCGAACCATCTTGTTGA
61
120
A L P T S N P A Q E L E A R Q L G R T T
pro-sequence
mature
NruI
BclI
Eco47III
XhoI
CGCGACGATCTGATCAACGGCAATAGCGCTTCCTGCGCCGATGTCATCTTCATTTATGCT
GCGCTGCTAGACTAGTTGCCGTTATCGCGAAGGACGCGGCTACAGTAGAAGTAAATACGA
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180
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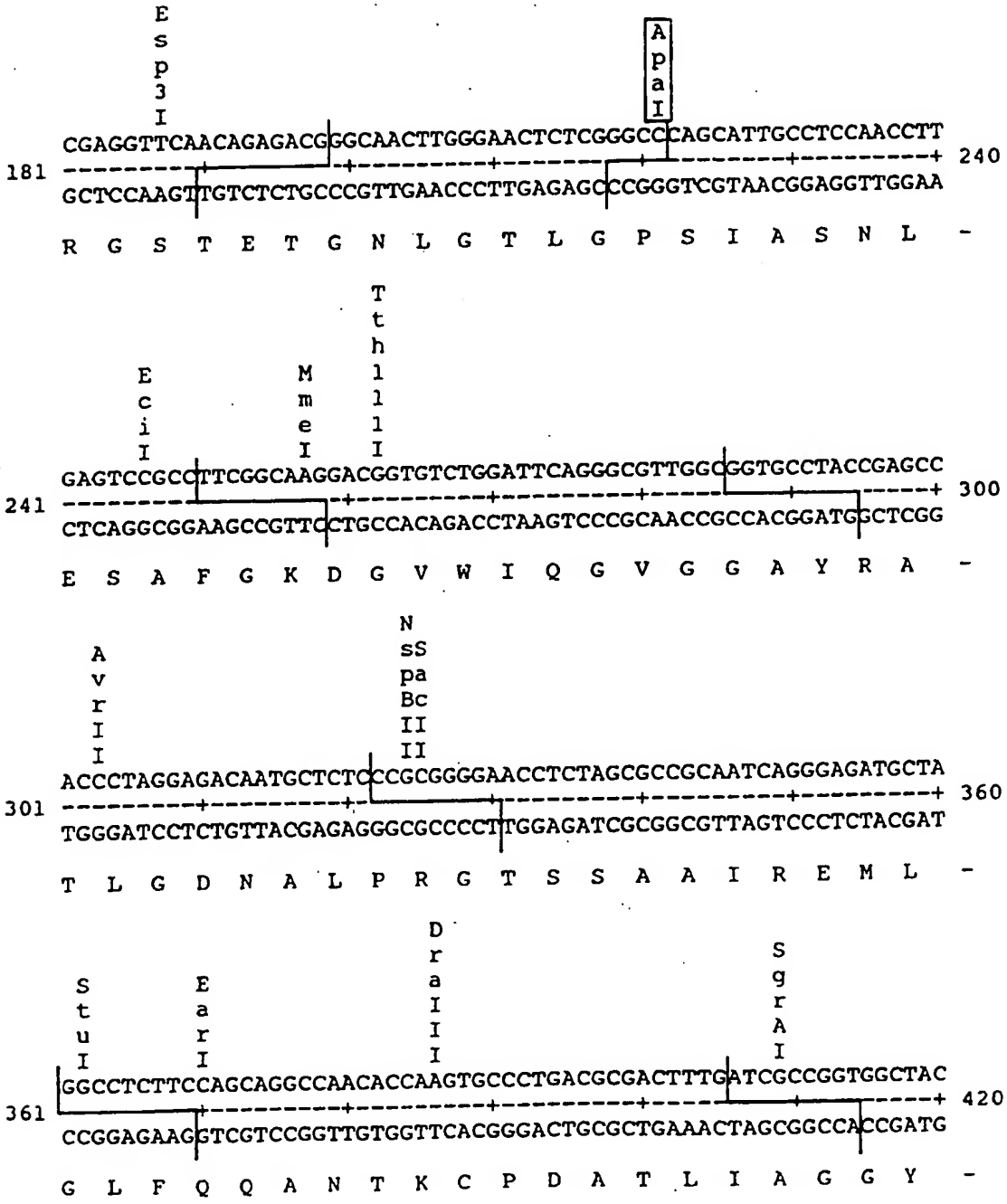
Fig.1D(2 of 4)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ApaI
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181
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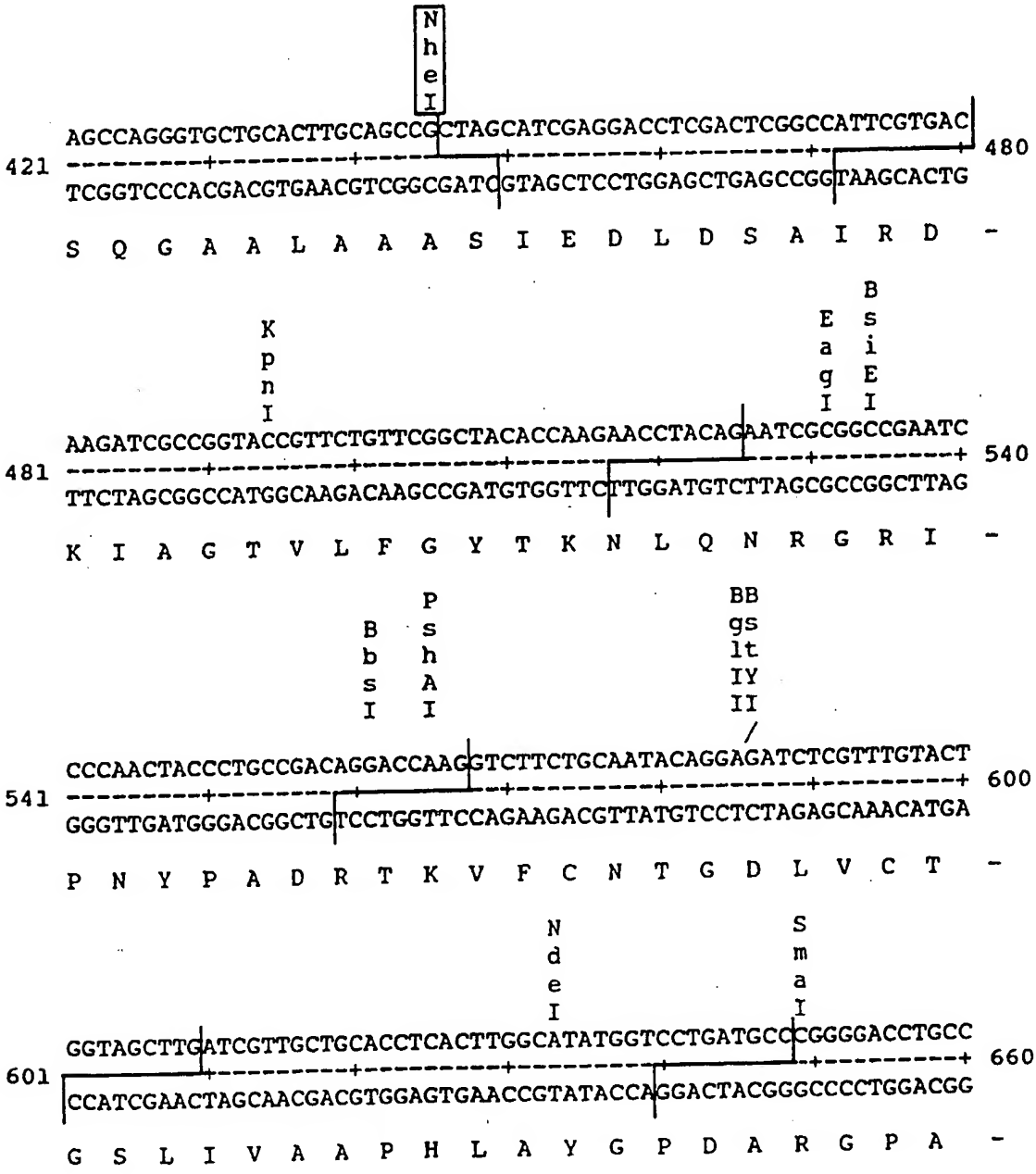
R G S T E T G N L G T L G P S I A S N L -
TthIII
EciI
MmeI
GAGTCCGCCTTCGGCAAGGACGGTGTCTGGATTCAGGGCGTTGGCGGTGCCTACCGAGCC
241
CTCAGGCGGAAGCCGTTCCTGCCACAGACCTAAGTCCCGCAACCGCCACGGATGGCTCGG 300
E S A F G K D G V W I Q G V G G A Y R A -
NnnSpaBcII
AvrII
ACCCTAGGAGACAATGCTCTCCCGCGGGGAACCTCTAGCGCCGCAATCAGGGAGATGCTA
301
TGGGATCCTCTGTTACGAGAGGGCGCCCCTTGGAGATCGCGGCGTTAGTCCCTCTACGAT 360
T L G D N A L P R G T S S A A I R E M L -
DraIII
StuI
EarI
SgrAI
GGCCTCTTCCAGCAGGCCAACACCAAGTGCCCTGACGCGACTTTGATCGCCGGTGGCTAC
361
CCGGAGAAGGTCGTCCGGTTGTGGTTCACGGGACTGCGCTGAAACTAGCGGCCACCGATG 420
G L F Q Q A N T K C P D A T L I A G G Y -

Fig.1D(3 of 4)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480
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S Q G A A L A A A S I E D L D S A I R D -
KpnI
BsiEI
EagI
AAGATCGCCGGTACCGTTCTGTTCGGCTACACCAAGAACCTACAGAATCGCGGCCGAATC
481
540
TTCTAGCGGCCATGGCAAGACAAGCCGATGTGGTTCTTGGATGTCTTAGCGCCGGCTTAG
K I A G T V L F G Y T K N L Q N R G R I -
PshAI
BbsI
BBgsltIYII
CCCAACTACCCTGCCGACAGGACCAAGGTCTTCTGCAATACAGGAGATCTCGTTTGTACT
541
600
GGGTTGATGGGACGGCTGTCCTGGTTCCAGAAGACGTTATGTCCTCTAGAGCAAACATGA
P N Y P A D R T K V F C N T G D L V C T -
NdeI
SmaI
GGTAGCTTGATCGTTGCTGCACCTCACTTGGCATATGGTCCTGATGCCCGGGGACCTGCC
601
660
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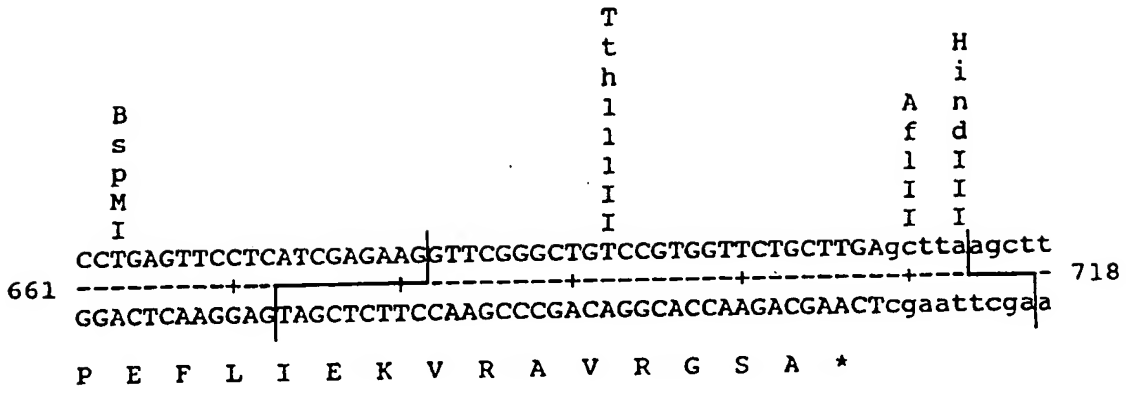
G S L I V A A P H L A Y G P D A R G P A -

Fig.1D(4 of 4)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AflIII
HindIII
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718
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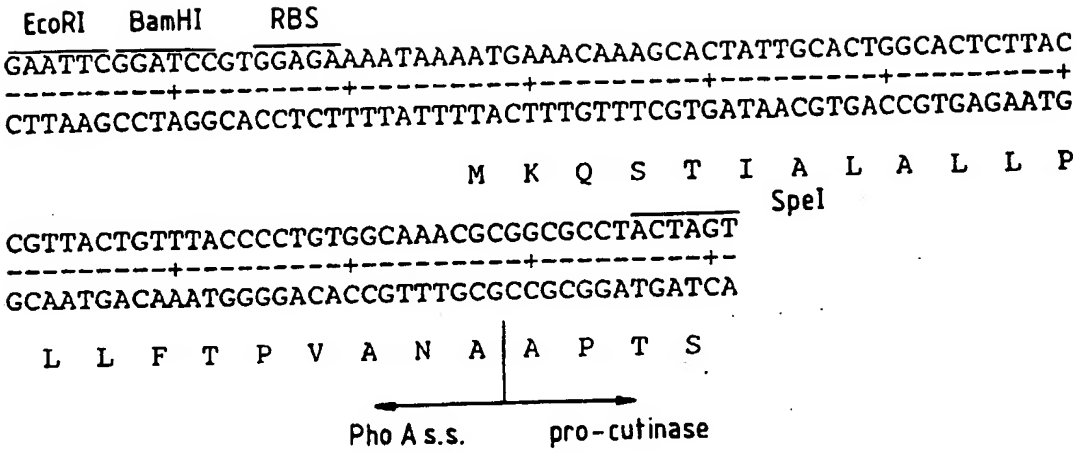
P E F L I E K V R A V R G S A *

Fig. 2.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BamHI
RBS
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M K Q S T I A L A L L P
SpeI
CGTTACTGTTTACCCCTGTGGCAAACGCGGCGCCTACTAGT
GCAATGACAAATGGGGACACCGTTTGCGCCGCGGATGATCA
L L F T P V A N A A P T S
Pho A s.s.
pro-cutinase

Fig. 3.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 8

CODE	Length	5' <--- sequence ---> 3'
		SacI
AC 01 cv	(38)	AAT TCT CGA GCT CAT CAC ACA AAC AAA CAA AAC AAA AT
AC 02 CV	(25)	GAT GCT TTT GCA AGC CTT CCT TTT C
AC 03 CV	(39)	CTT TTG GCT GGT TTT GCA GCC AAA ATA TCT GCG GGT AGA
		BclI
AC 04 CV	(25)	ACA ACT CGC GAC GAT CTG ATC ATC A
AC 05 CV	(41)	AGC TTG ATG ATC AGA TCG TCG CGA GTT GTT CTA CCC GCA GA
AC 06 CV	(17)	TAT TTT GGC TGC AAA AC
AC 07 CV	(46)	CAG CCA AAA GGA AAA GGA AGG CTT GCA AAA GCA TCA TTT TGT TTT G
AC 08 CV	(23)	TTT GTT TGT GTG ATG AGC TCG AG

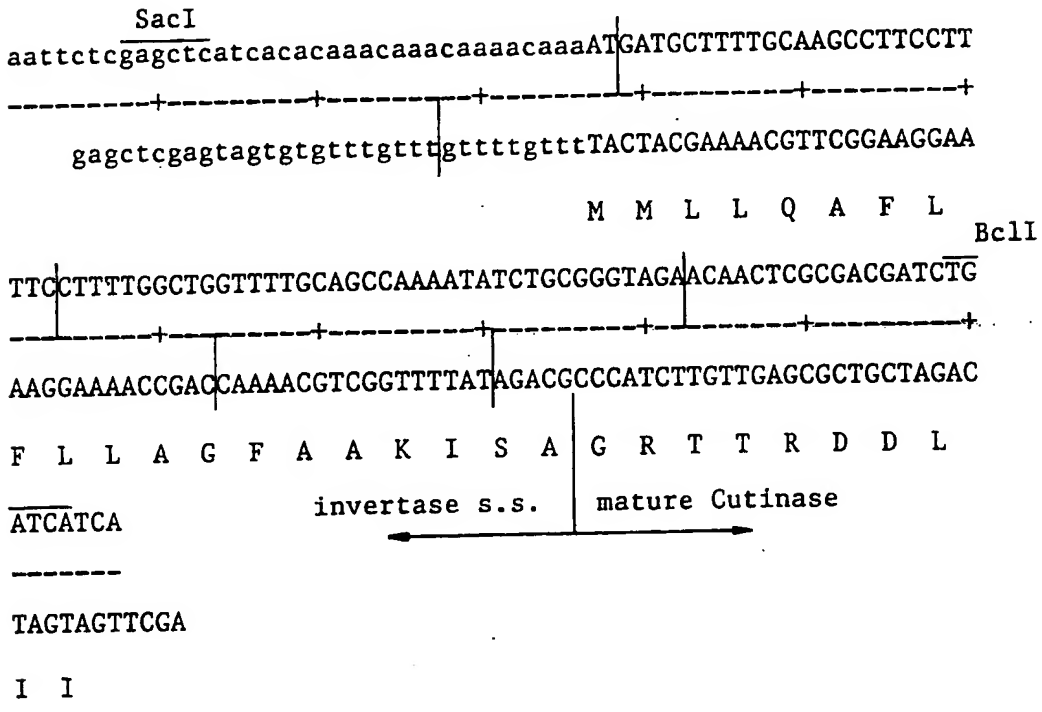


Fig.4.

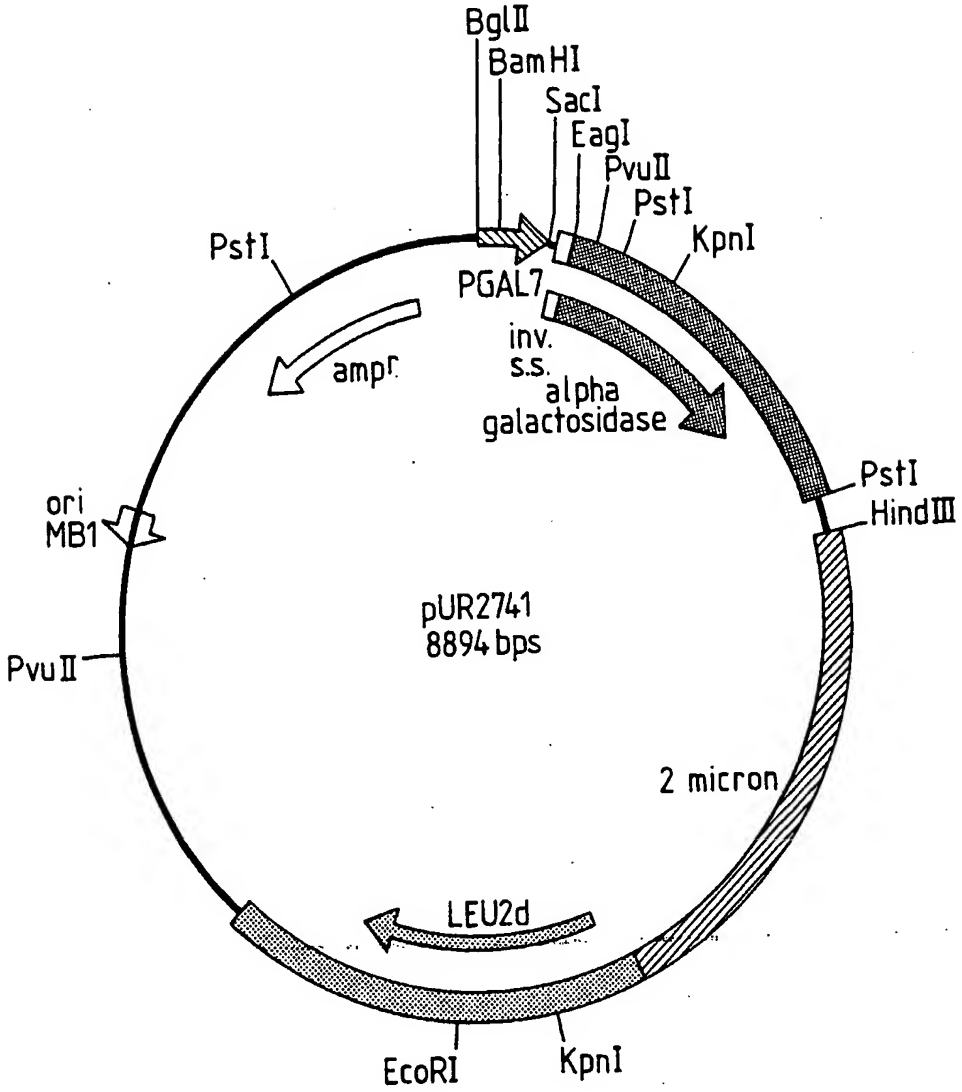
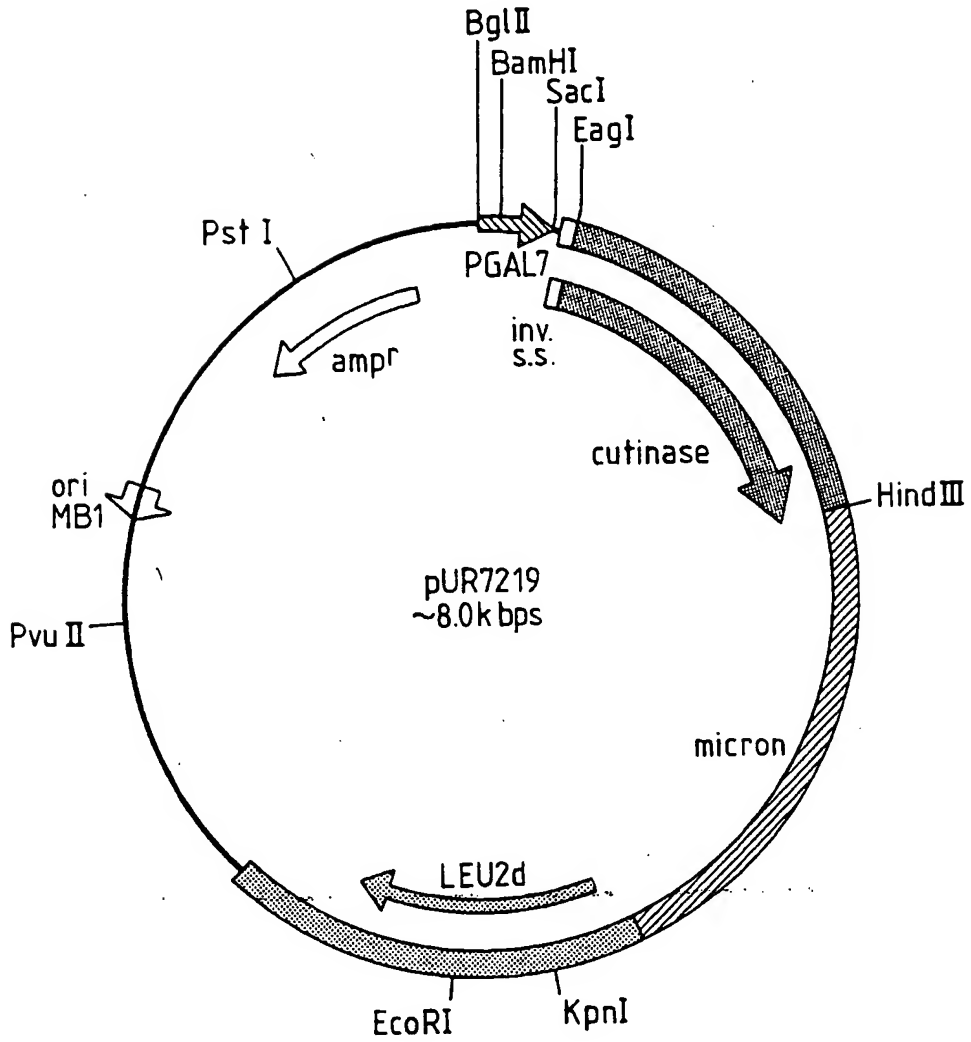

BglII
BamHI
SacI
EagI
PvuII
PstI
KpnI
PstI
PGAL7
inv.
s.s.
alpha
galactosidase
ampr
PstI
HindIII
ori
MB1
pUR2741
8894 bps
PvuII
2 micron
LEU2d
EcoRI
KpnI

Fig. 5.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Pst I
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inv.
s.s.
ampr
cutinase
HindIII
ori
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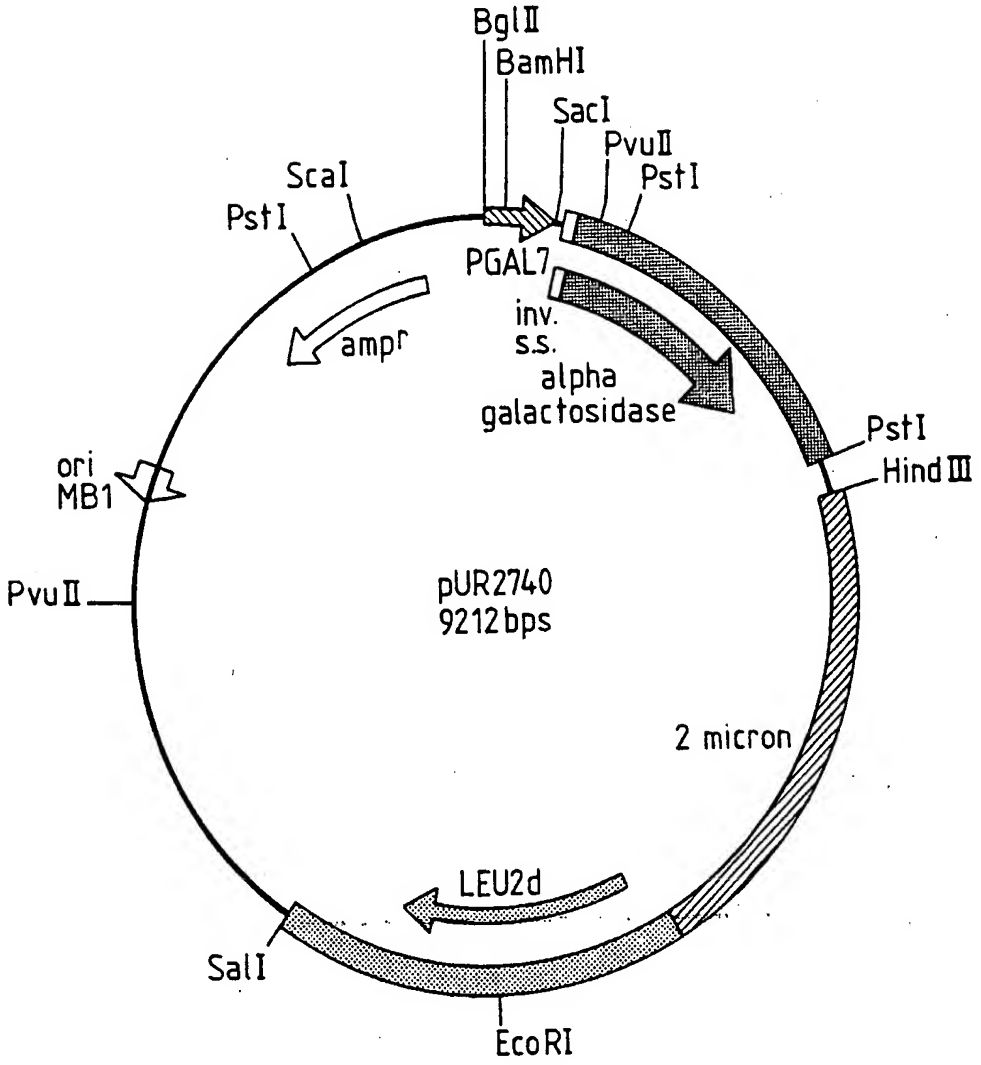
pUR7219
~8.0k bps
Pvu II
micron
LEU2d
EcoRI
KpnI

Fig. 6.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BamHI
SacI
PvuII
PstI
ScaI
PstI
PGAL7
inv.
s.s.
alpha
galactosidase
ampr
PstI
HindIII
ori
MB1
PvuII
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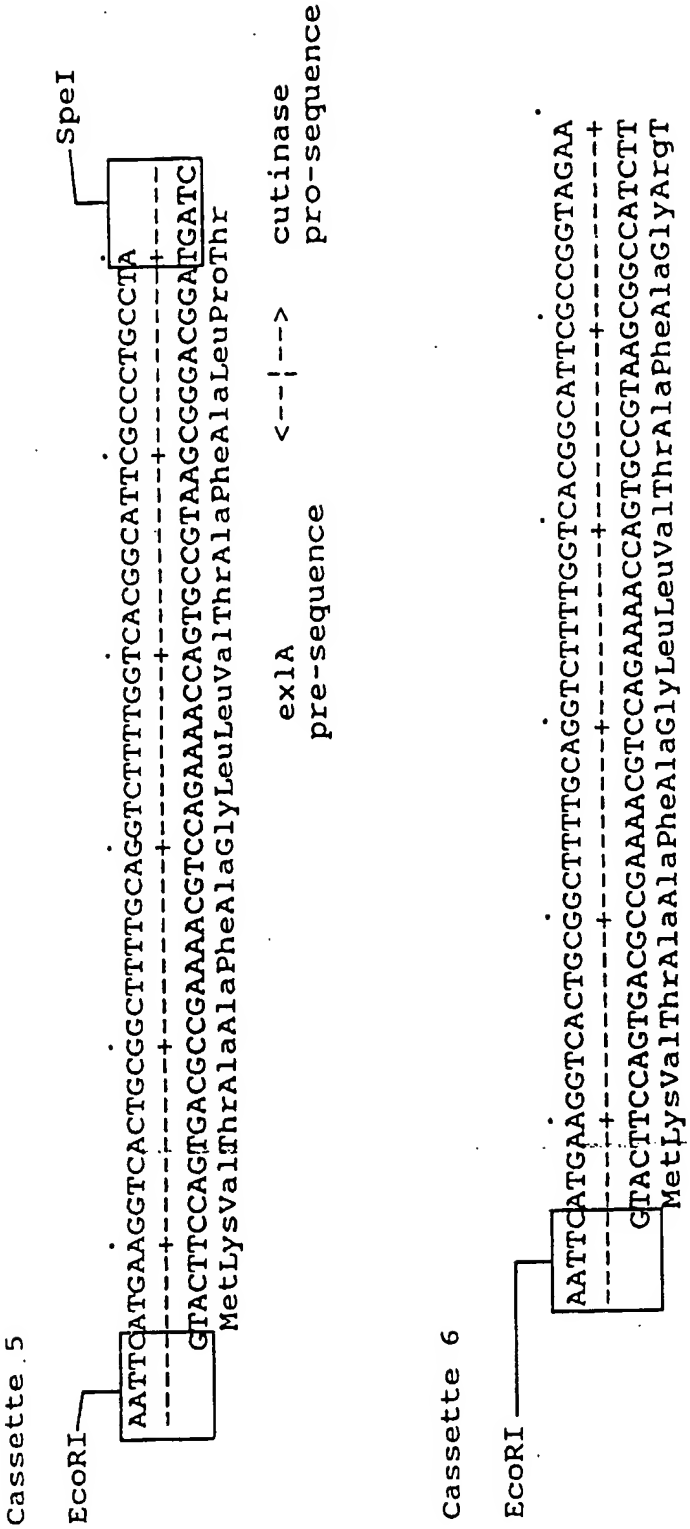
9212bps
2 micron
LEU2d
SalI
EcoRI

Fig. 7.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SpeI
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MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaLeuProThr
ex1A
pre-sequence
<--¦-->
cutinase
pro-sequence
Cassette 6
EcoRI
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GTACTTCCAGTGACGCCGAAAAACGTCCAGAAAACCAGTGCCGTAAGCGGCCCATCTT
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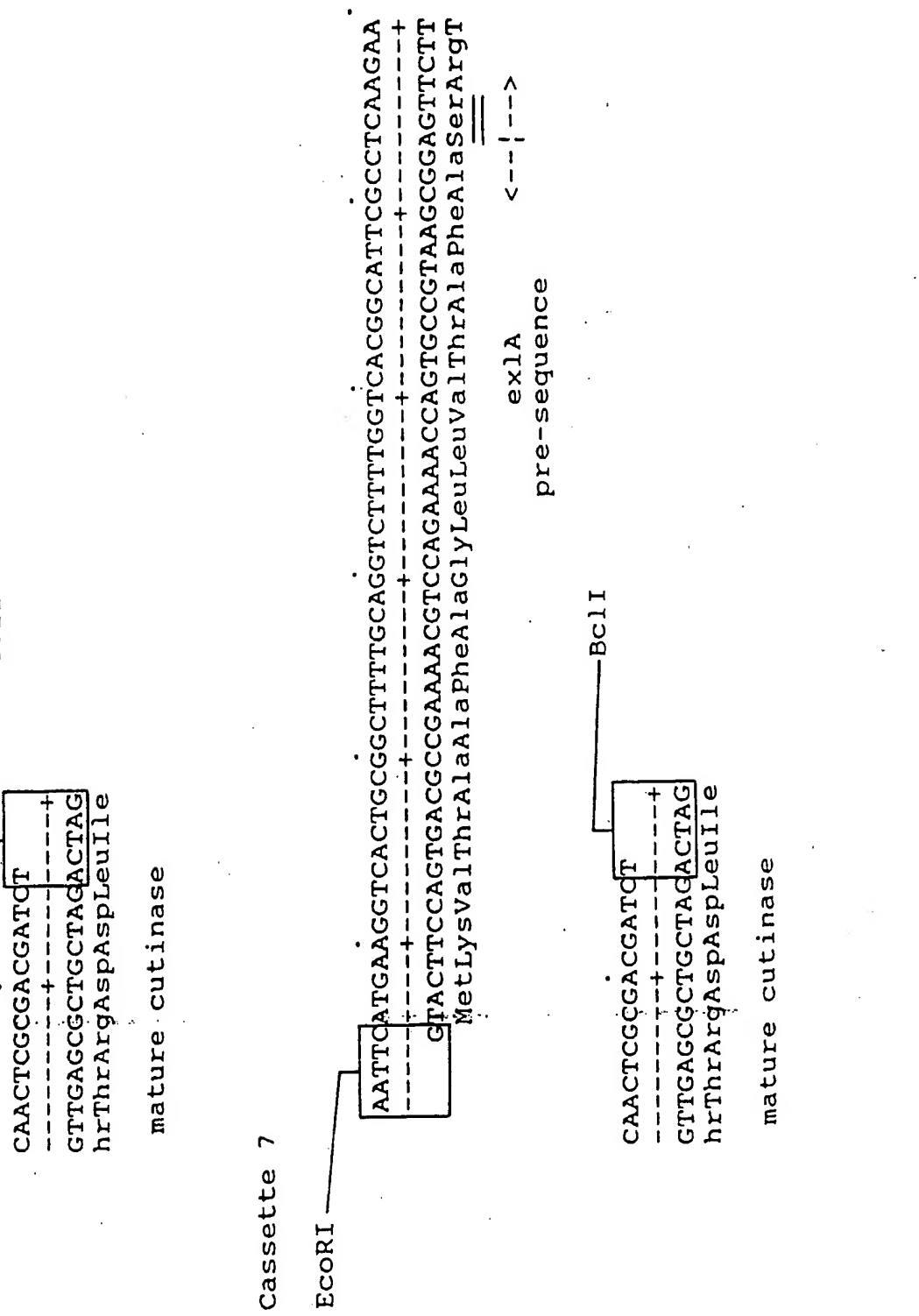
ex1A
pre-sequence
<--¦-->

Fig. 7(Cont).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mature cutinase
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MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaSerArgT
exlA
pre-sequence
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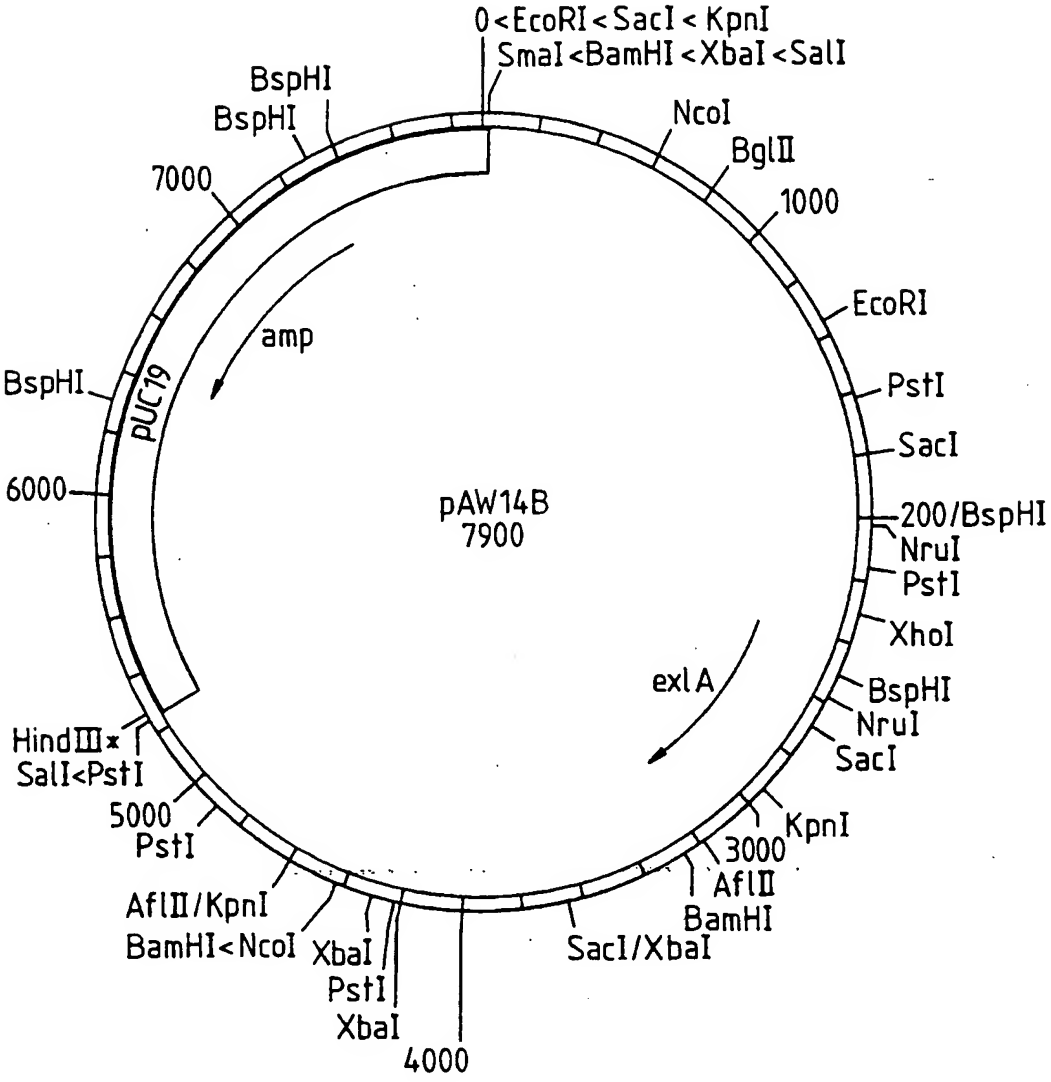
mature cutinase

Fig. 8.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SmaI<BamHI<XbaI<SalI
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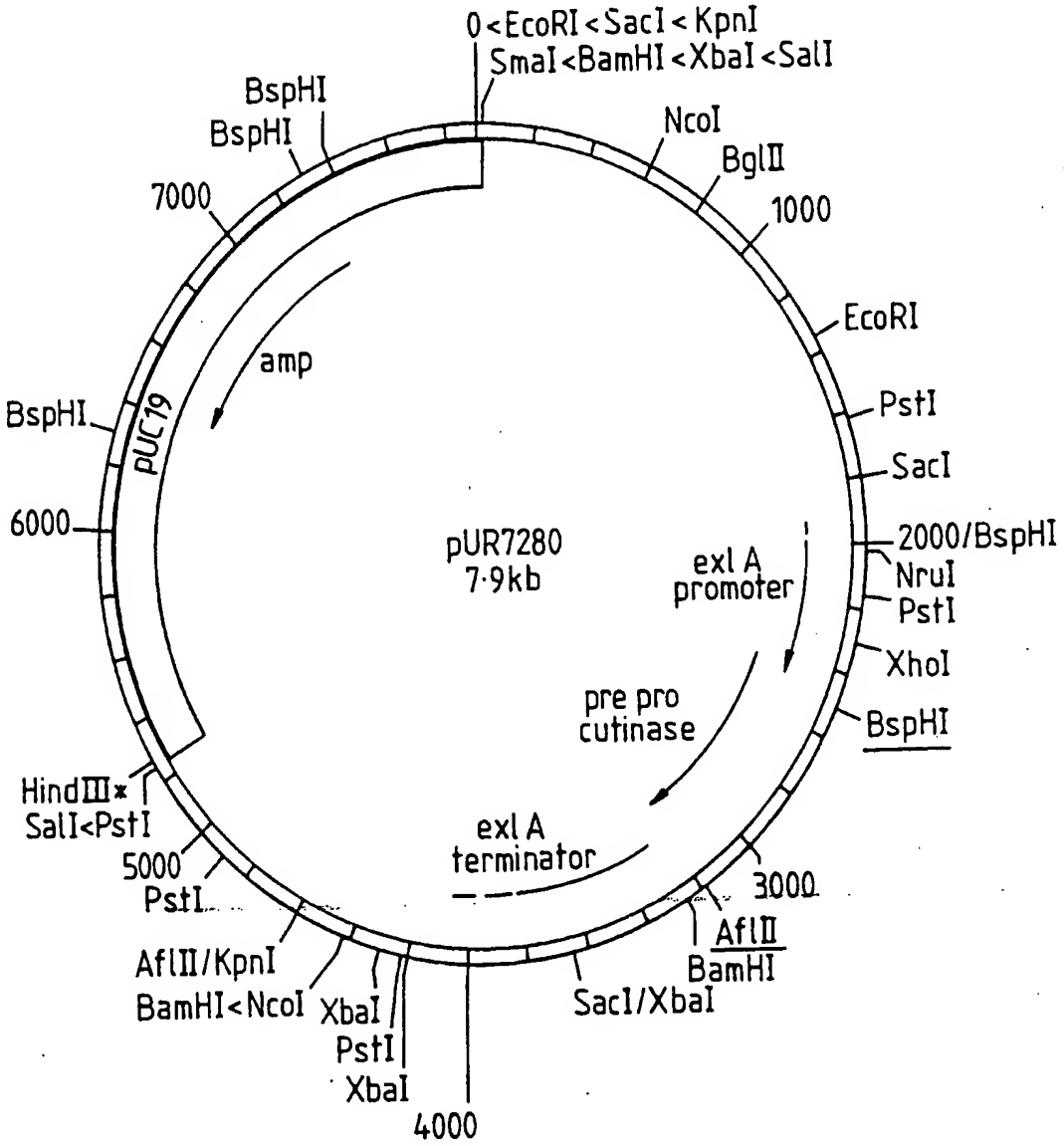
BspHI
NcoI
BglII
1000
7000
EcoRI
amp
BspHI
pUC19
PstI
SacI
6000
pAW14B
7900
200/BspHI
NruI
PstI
XhoI
exlA
BspHI
NruI
SacI
HindIII
SalI<PstI
5000
PstI
KpnI
3000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
XbaI
4000
SacI/XbaI

Fig. 9.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PstI
SacI
2000/BspHI
NruI
PstI
XhoI
BspHI
7000
amp
pUC19
BspHI
6000
pUR7280
7·9kb
exl A
promoter
pre pro
cutinase
exl A
terminator
HindIII*
SalI<PstI
5000
PstI
AflII/KpnI
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XbaI
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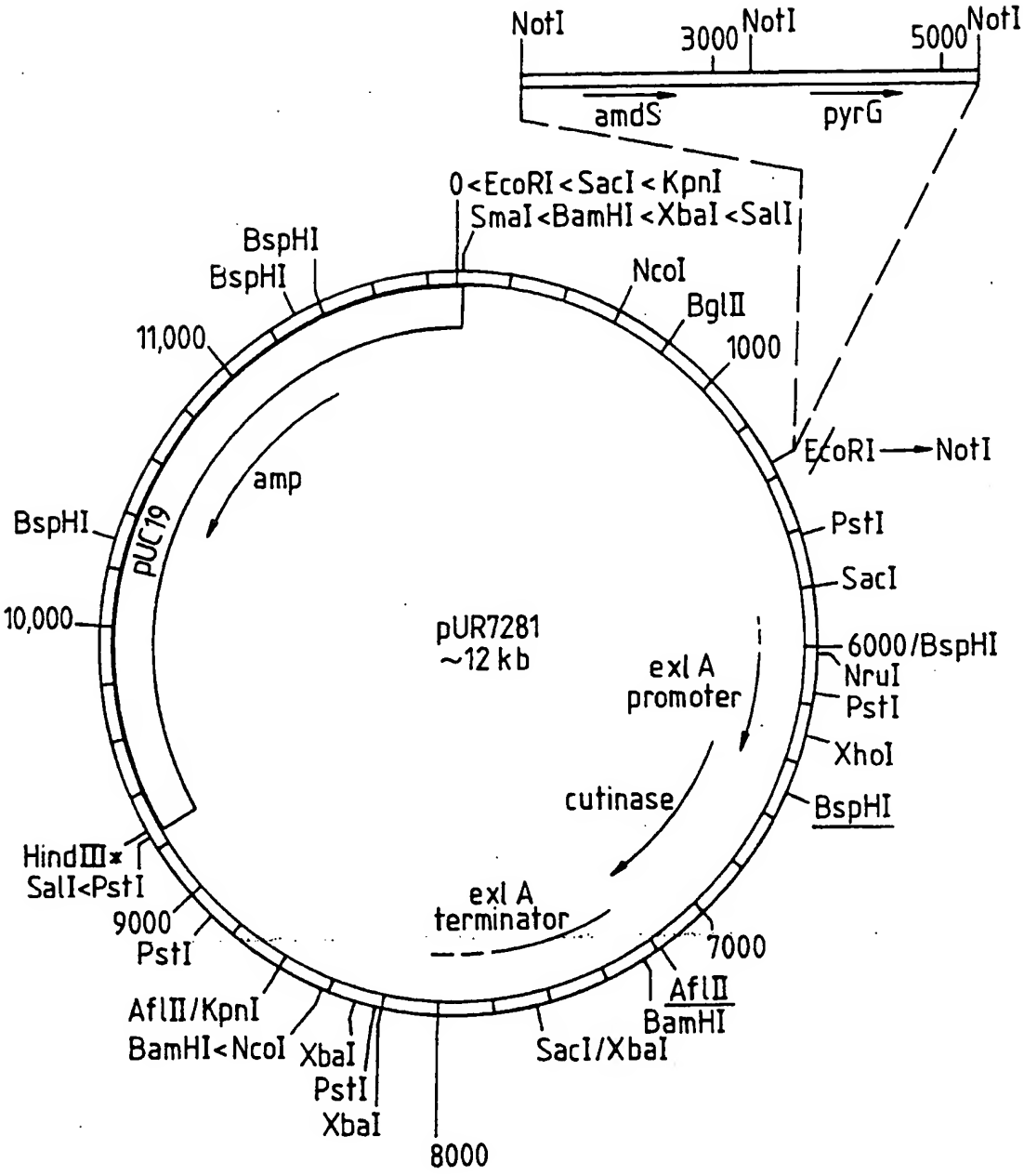
SacI/XbaI
3000
AflII
BamHI

Fig.10.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NotI
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NotI
amdS
pyrG
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BspHI
BspHI
NcoI
BglII
1000
11,000
EcoRI → NotI
PstI
SacI
6000/BspHI
NruI
PstI
XhoI
BspHI
amp
pUC19
BspHI
10,000
pUR7281
~12 kb
exl A
promoter
cutinase
exl A
terminator
HindIII
SalI<PstI
9000
PstI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
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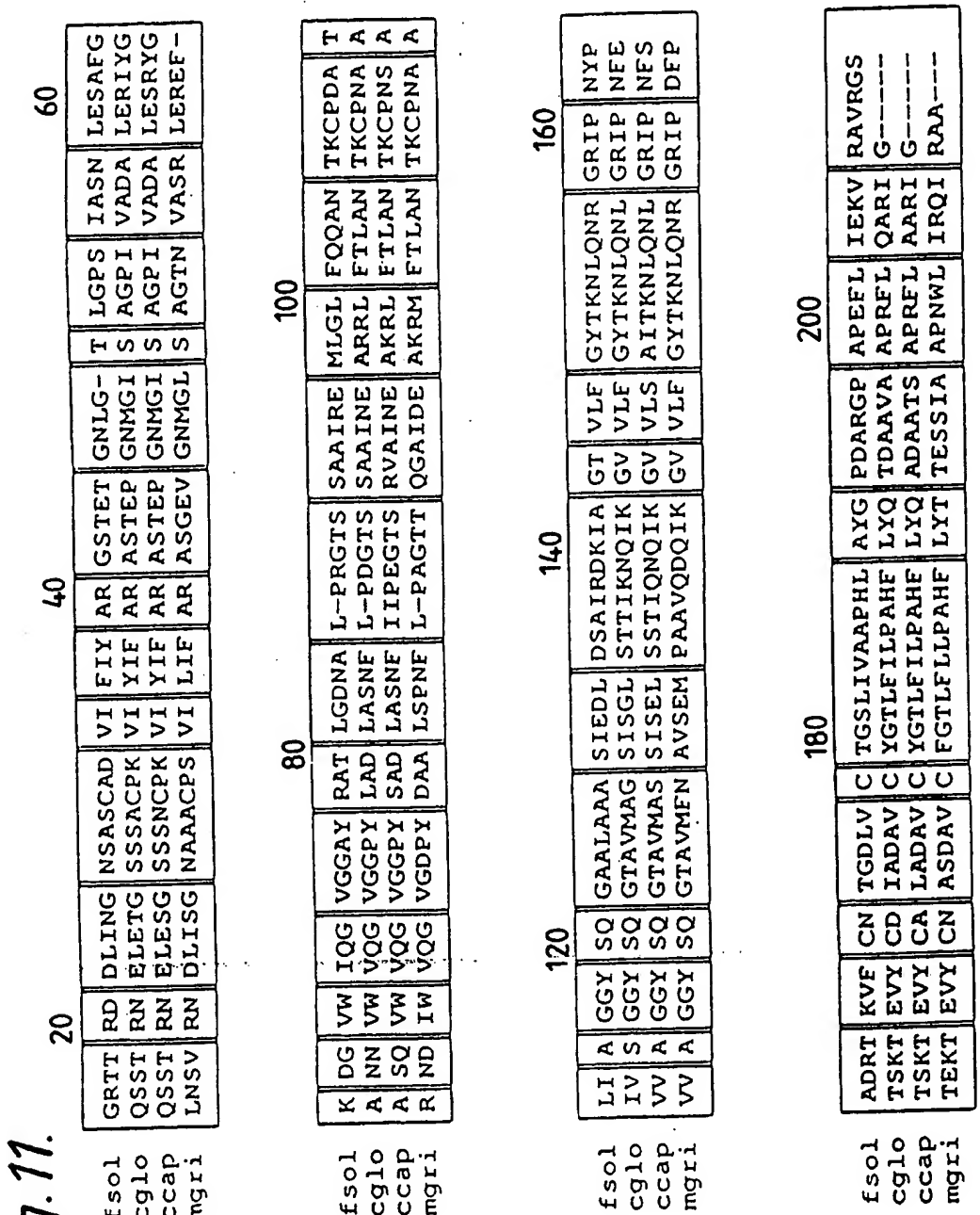
SacI/XbaI
7000
AflII
BamHI

Fig. 11.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40
60
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ccap QSST RN ELESG SSSNCPK VI YIF AR ASTEP GNMGI S AGPI VADA LESRYG
mgri LNSV RN DLISG NAAACPS VI LIF AR ASGEV GNMGL S AGTN VASR LEREF-
80
100
fsol K DG VW IQG VGGAY RAT LGDNA L-PRGTS SAAIRE MLGL FQQAN TKCPDA T
cglo A NN VW VQG VGGPY LAD LASNF L-PDGTS SAAINE ARRL FTLAN TKCPNA A
ccap A SQ VW VQG VGGPY SAD LASNF IIPEGTS RVAINE AKRL FTLAN TKCPNS A
mgri R ND IW VQG VGDPY DAA LSPNF L-PAGTT QGAIDE AKRM FTLAN TKCPNA A
120
140
160
fsol LI A GGY SQ GAALAAA SIEDL DSAIRDKIA GT VLF GYTKNLQNR GRIP NYP
cglo IV S GGY SQ GTAVMAG SISGL STIKNQIK GV VLF GYTKNLQNL GRIP NFE
ccap VV A GGY SQ GTAVMAS SISEL SSTIQNQIK GV VLS AITKNLQNL GRIP NFS
mgri VV A GGY SQ GTAVMFN AVSEM PAAVQDQIK GV VLF GYTKNLQNR GRIP DFP
180
200
fsol ADRT KVF CN TGDLV C TGSLIVAAPHL AYG PDARGP APEFL IEKV RAVRGS
cglo TSKT EVY CD IADAV C YGTLFILPAHF LYQ TDAAVA APRFL QARI G------
ccap TSKT EVY CA LADAV C YGTLFILPAHF LYQ ADAATS APRFL AARI G------
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Fig. 12.

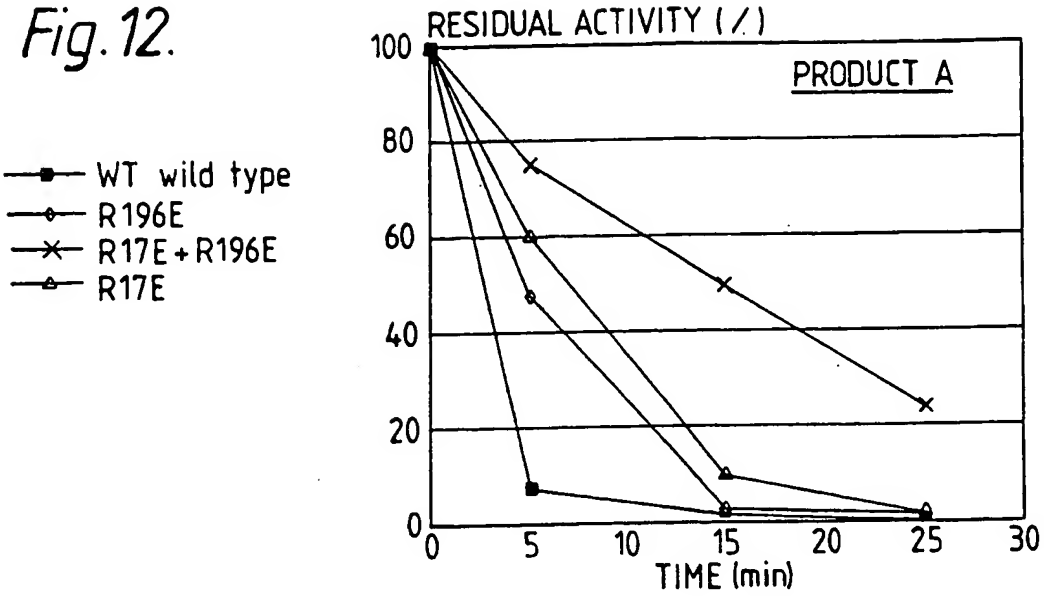
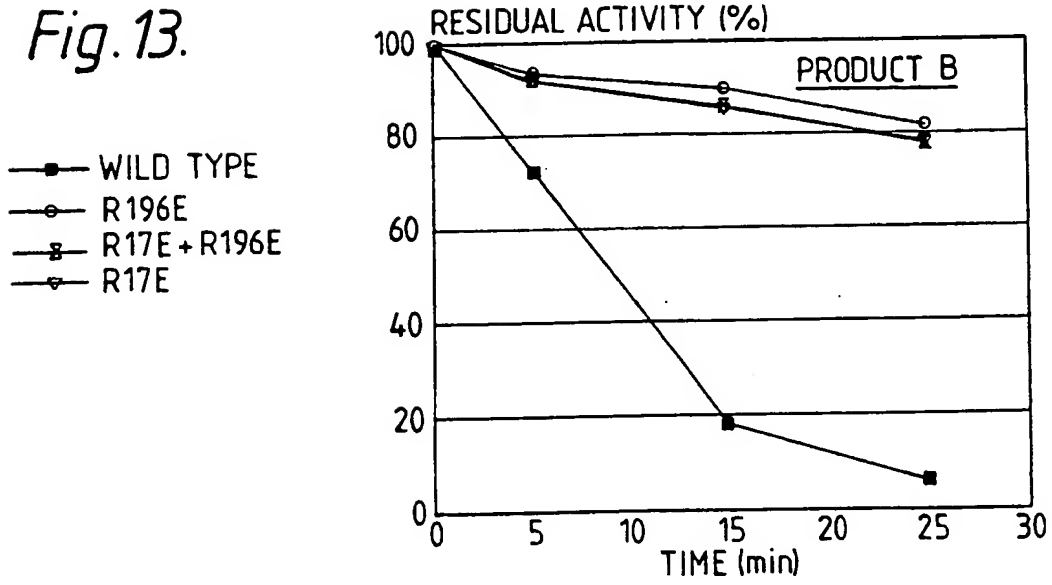

WT wild type
R196E
R17E + R196E
R17E
RESIDUAL ACTIVITY (/)
PRODUCT A
100
80
60
40
20
0
0
5
10
15
20
25
30
TIME (min)

Fig. 13.


WILD TYPE
R196E
R17E + R196E
R17E
RESIDUAL ACTIVITY (%)
PRODUCT B
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80
60
40
20
0
0
5
10
15
20
25
30
TIME (min)

Fig. 14.

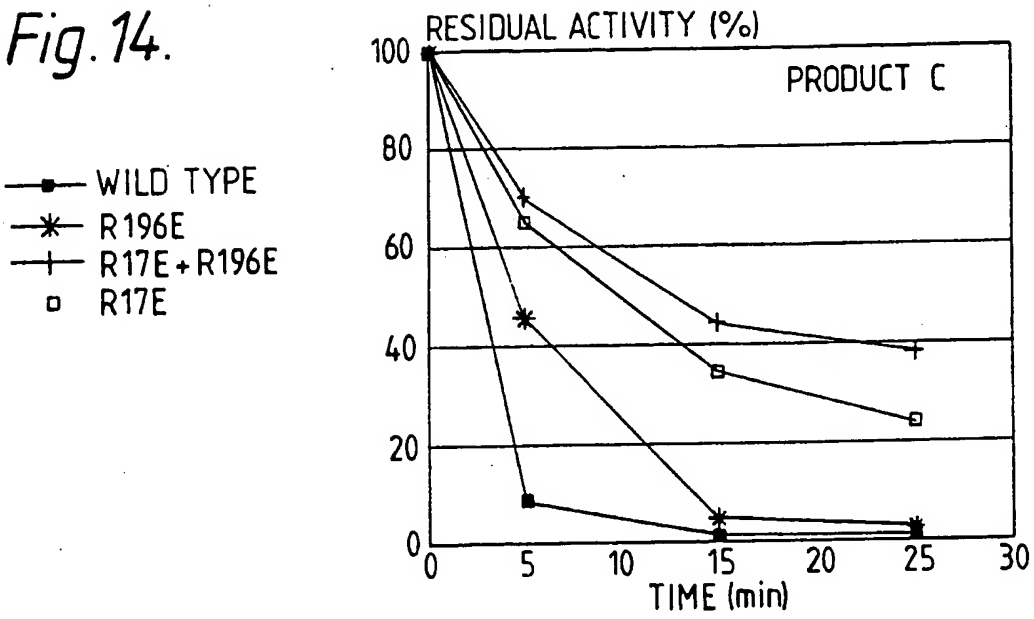
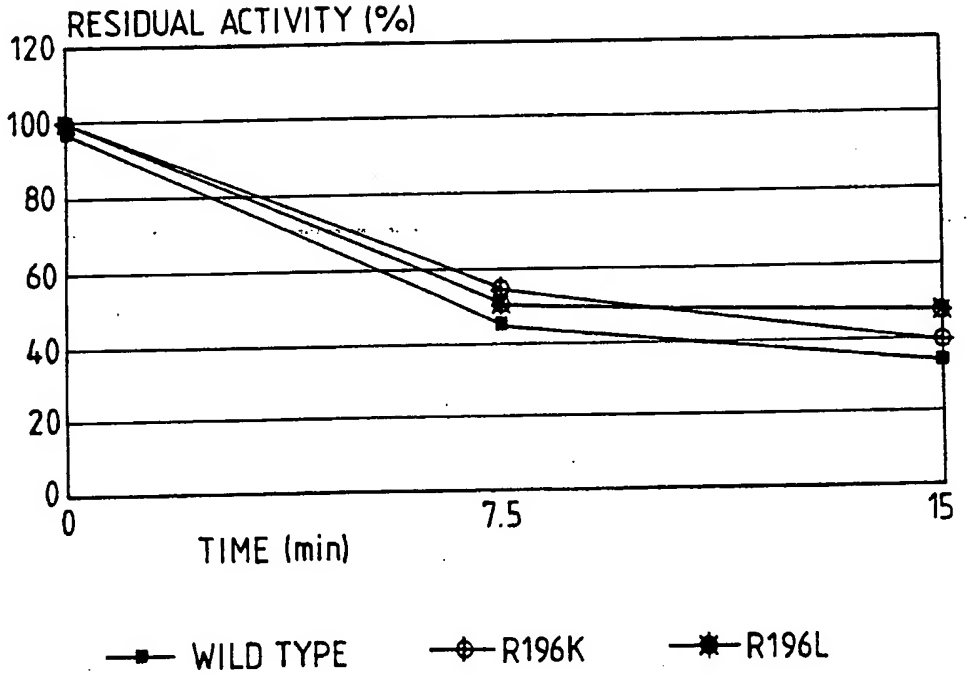

WILD TYPE
R196E
R17E+R196E
R17E
RESIDUAL ACTIVITY (%)
PRODUCT C
100
80
60
40
20
0
0
5
10
15
20
25
30
TIME (min)

Fig. 15.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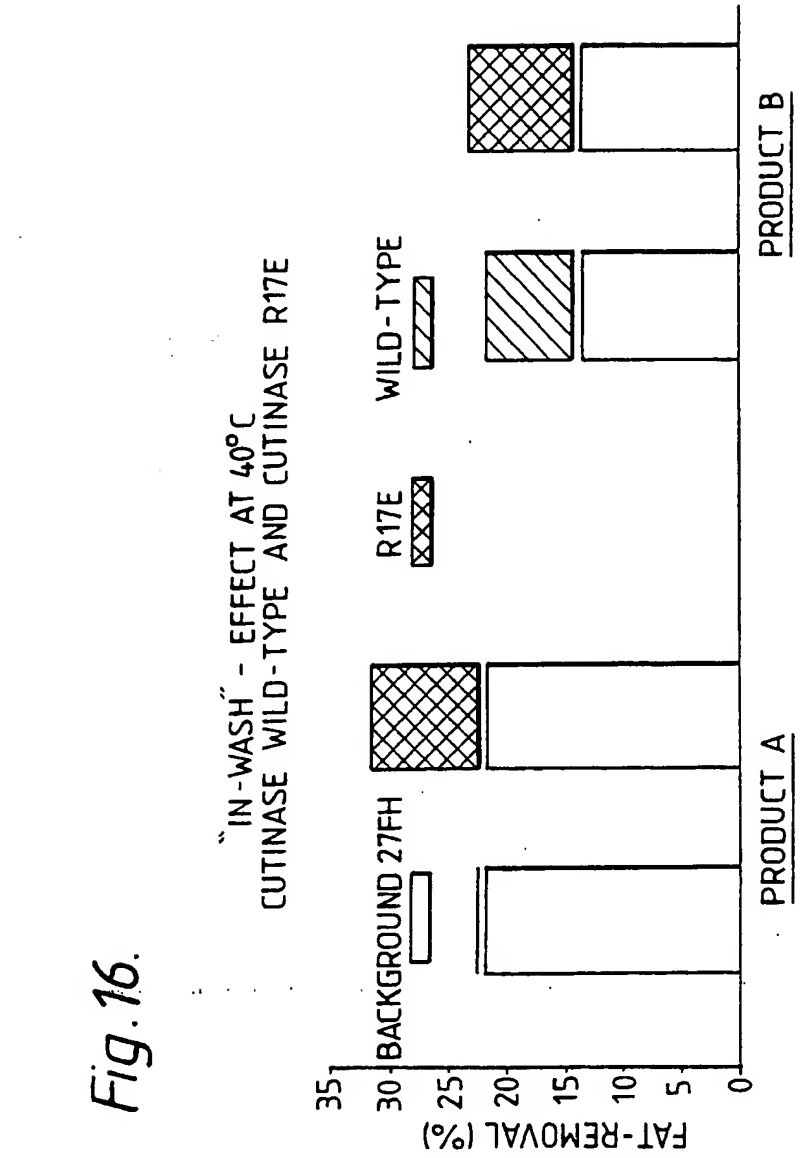
100
80
60
40
20
0
0
7.5
15
TIME (min)
WILD TYPE
R196K
R196L

Fig. 16.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CUTINASE WILD-TYPE AND CUTINASE R17E
FAT-REMOVAL (%)
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30
25
20
15
10
5
0
BACKGROUND 27FH
R17E
WILD-TYPE
PRODUCT A
PRODUCT B